

After Toronto

Effective AIDS prevention requires far better understanding of why existing strategies do not succeed.

At the AIDS meeting that has just ended in Toronto, the resounding theme was a fresh emphasis on prevention of the disease. The reasons for this are straightforward. A lot of work has been done to get antiretroviral therapies out to 1.3 million people with HIV, but that is still only one-fifth of the people who need them. And for every person put on life-saving treatments each year, there are ten new infections.

Some promising prevention strategies are edging from the laboratory to the clinic. Trials on microbicides, which could help protect women whose partners won't use a condom, are expected to start delivering results late next year. One large trial has already found that male circumcision may cut the risk of HIV infection by 60%, and others are attempting to confirm this finding. Another study has established that antiretroviral drugs are safe for daily dosing in people without HIV, paving the way for larger tests of whether this could protect people from infection. All these approaches have limitations but are worthy of fuller exploration.

Additionally, an emerging move towards large-scale collaboration could bolster the efficiency of HIV research. Microbicide researchers are already communicating closely through several formal and informal mechanisms, and a roadmap was set out for this in a Microbicide Development Strategy, released on 17 August.

Vaccine researchers have also recently been required to collaborate more closely, under the umbrella of the Global HIV Vaccine Enterprise, supported by the Bill & Melinda Gates Foundation. Such collaboration has unfortunately been rare since the early days of HIV vaccine research. It may help to tackle some of the frustrations and dead-ends that have characterized the field from the start.

Individual HIV researchers have also begun banding together to counter divisive tendencies that they say have held the field back. Bruce Walker of Harvard Medical School has announced the beginning of a study on people who maintain good health despite being infected with HIV (see page 852). By studying these 'elite controllers',

Walker and his colleagues aim to discover why these patients are able to conquer the virus, whereas others cannot. That information could guide a more effective vaccine. Walker says the study will publish its results under a group name — the HIV Elite Controllers Consortium — with no first or senior author. This is a conscious attempt to break away from the divisions and rivalries that have previously dogged some research teams.

The approach is encouraging. But one of the most quoted statistics at the AIDS meeting was that one in five people worldwide at high risk of HIV infection don't have access to prevention practices that already exist. This is partly a resource problem: according to Beatrice Were of ActionAid Uganda, there are only three condoms per year available for every man in southern Africa.

But the failure of prevention strategies is also a scientific problem. On 17 August, a team from the World Health Organization and Johns Hopkins Bloomberg School of Public Health in Baltimore, Maryland, described their efforts to survey the published literature to establish what works for AIDS prevention in poor countries. The interventions studied included harm-reduction strategies for drug users who inject, targeted education programmes and abstinence. The meta-analysis found compelling evidence only for harm reduction — the strategy that political leaders, particularly in the United States, are least willing to fund.

The survey's main conclusion was that there remains a paucity of reliable data on the effectiveness of prevention strategies in developing countries. More epidemiologists and social scientists need to focus their energies on testing appropriate prevention methods in the places where the AIDS epidemic is at its worst. It is hopeless to await success with microbicides, or other biomedical strategies, if we don't even know why current interventions are failing. ■

"One in five people worldwide at high risk of HIV infection don't have access to prevention practices that already exist."

State of readiness

The anniversary of Hurricane Katrina should remind scientists to keep disaster recovery plans in order.

This week marks the first anniversary of Hurricane Katrina's devastating visit to the US Gulf Coast. The images associated with the disaster are well-known: the anguish of New Orleans residents trapped at the Superdome as rescue teams rafted from house to house, finding mainly corpses.

But researchers would do well to recall a Katrina image of their own: that of a convoy of sports utility vehicles, escorted by armed guards, that descended on university buildings after the disaster.

Emergency workers were able to salvage some important biomedical data, retrieving important laboratory animals and thrusting cell cultures and tissue specimens into temporary refrigeration.

At several institutions in the city, however, including the health-sciences centre at Tulane University (see page 856), key research materials were lost. What wasn't flooded by Katrina's waters was doomed by power failures in the stifling August heat. Back-up generators, where they existed, were often in flood-prone basements.

The pattern of loss echoed an experience in Houston, Texas, in 2001, when Tropical Storm Allison swept ashore, flooding low-lying buildings. Dozens of monkeys and dogs were drowned at the University of Texas Medical Center at Houston. Last year, a similar fate befell 8,000 laboratory animals at the Louisiana State University Medical Center in New Orleans. Many drowned in the floodwaters;



others starved or had to be killed later. Unfortunately, the obvious lessons from Allison had not been applied in New Orleans in time for Katrina.

Relatively simple precautions can often prepare laboratories to deal with natural disasters, especially in regions where the risk is known to be high. In earthquake-prone San Francisco, researchers typically know how well their buildings are constructed to withstand a quake, and sometimes practise procedures for evacuation in such an event. In New Orleans, researchers had lived with the threat of flooding for years. Yet the threat was perceived as indeterminate — everything, it seemed, would be fine, except in the event of a levee breaking. Of course, the levee broke.

Subsequent events should remind scientists in regions where such risks exist to revisit their own laboratories' emergency preparations. Researchers who work with animals should prepare a tagging system to help them identify the animals most crucial to their work. In the case of power outages, rescuers can identify those animals through

their brightly coloured tags and know to take them out first.

Those with crucial cell lines in need of refrigeration should make sure that back-up systems are in place to keep the samples cold. Dry-ice can serve to keep precious samples refrigerated, even if the power is out for several days.

For most organizations, maintaining communications will be the most critical aspect of disaster recovery. Laboratories should ensure they have up-to-date telephone numbers for all members of the lab, and a system in place for who should contact whom in an emergency. E-mail systems should be backed up on remote servers, so they can be kept running throughout. These steps assume, of course, that some infrastructure will continue to function within a nearby community, where evacuees can regroup.

Such preparations rarely take priority until disaster strikes. But every researcher, from lab head to summer student, should look at what surrounds them, apply some common sense, and acquaint themselves with the basics of disaster recovery for their laboratory. ■

Foo's paradise

In praise of chat.

It's not uncommon to hear despairing complaints about some high-powered meeting that it was 'nothing more than a talking shop'. If one is going to all the trouble of gathering these people, the accusation suggests, it should deliver something.

That's a fair complaint when hard-edged achievement is the avowed intention. But maybe too little credence is given to gatherings that are expressly intended to foster conversation, organized in the enlightened hope that people will be stimulated and that unanticipated developments will follow. The duty of the organizers, then, is simply to maximize the chances of positive encounters.

Some years ago, the publisher Tim O'Reilly and his colleagues conceived the idea of just such a talkfest. O'Reilly is an influential enthusiast of participative web activities such as wikis and blogs. And in the same spirit, the programme of such meetings is developed on the spot by the 100-odd participants, who arrive at some enjoyable location and camp together for three days. The initial idea was to invite 'friends of O'Reilly', and thus was the first 'Foo camp' conceived. True to form, no grand manifestos or initiatives have emerged, but there has been plenty of stimulation and, no doubt, some upward blips in the revenues of manufacturers of alcoholic beverages.

Given the interest of *Nature* and its publishers in participatory publishing — see, for example, the strings of comments on some of the news stories of news@nature.com and our trial of open peer review (<http://blogs.nature.com/nature/peerreview/trial/>) — it was no surprise that we should fraternize with O'Reilly and conceive the idea of a science Foo. And it was gratifying that Google thought the idea sufficiently fun to be worth hosting such a get-together. And so it was that 200 people — scientists, mainly, infused with technologists and writers — turned up at the 'Googleplex' in Mountain View, California, earlier this month, for a long weekend of chat.

Invitees, who ranged across disciplines, age and nationality, were not told who else was coming. They were simply invited to get

themselves there. Formal presentations were not encouraged. And the key to the dynamic was the programme: a wall chart with an empty matrix of one-hour sessions in a number of variously sized rooms stretching across two-and-a-half days, each session to be specified by any individual attendee as the meeting progressed. (It was fun to see who rated their session as worthy of 150 people's attention, and who offered their topic to a mere eight.)

After an introductory session, the participants developed a programme of titles such as 'how to radicalize scientists', 'open peer review and science wikis', 'the future of human evolution', 'text mining', 'educational robotics', 'global health', 'the semantic web and the life sciences', and 'citizen scientists' (which features in this week's *Nature* podcast).

Inevitably such meetings will pick up on common concerns of the moment — such as the relationship between science and politics, how scientists should deal with fallacious media coverage, the balance between open and proprietary approaches to anything and everything. But there were plenty of uncommon ideas too, such as putting an atmospheric sensor on every mobile phone, and analysing the 'parameter space' of sciences and technologies in order to map and anticipate future advances.

The exercise could be portrayed by cost-conscious administrators as a colossal act of self-indulgence. But, for example, an entrepreneur who wants to design a \$20 float for an oceanography experiment got some ideas about how to move his project forward, and an advocate of public participation in clinical trials was given feedback on her plans to use home DNA kits to boost involvement in a cancer trial. Many attendees commented on the stimulation of getting feedback on their ideas from an unusual mix of expertise.

But above all, it was the mode of spontaneous organization that gave the meeting a drive that is unusual and worth promoting. If this is what a talking-shop can be like, let's have more of them. ■

"Many attendees commented on the stimulation of getting feedback on their ideas from an unusual mix of expertise."

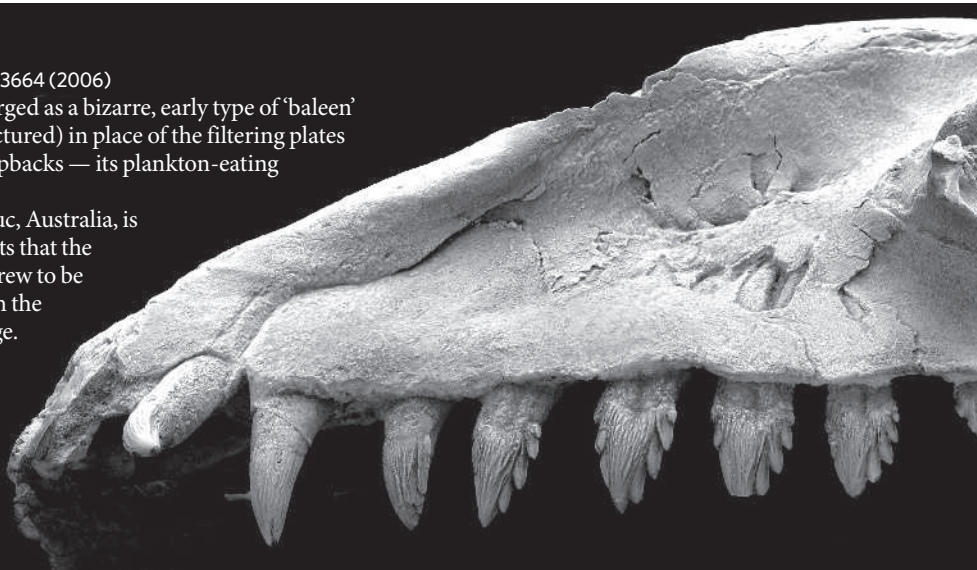
RESEARCH HIGHLIGHTS

Nice gnashers

Proc. R. Soc. Lond. B doi:10.1098/rspb.2006.3664 (2006)

A 25-million-year-old fossil has emerged as a bizarre, early type of 'baleen' whale. It has a set of vicious teeth (pictured) in place of the filtering plates used by today's blue whales and humpbacks — its plankton-eating relatives.

The fossil skull, discovered in Jan Juc, Australia, is only 50 centimetres long. This suggests that the species, dubbed *Janjucetus hunderi*, grew to be a few metres in length, compared with the 30 metres that blue whales can manage. Certain features of the skull, such as the way the snout meets the brain case, identify it as a relative of baleen whales (Mysticeti), reports Erich Fitzgerald of Monash University in Clayton, Australia. Predatory species, it seems, evolved alongside filter feeders.



R. START/MUSEUM VICTORIA

CELL BIOLOGY

Acid bath

Nature Cell Biol. doi:10.1038/ncb1456 (2006)

Researchers think they may be able to explain why cystic-fibrosis patients are extremely vulnerable to severe infection in the lungs, but not elsewhere in the body.

Cystic fibrosis is caused by defects in a membrane protein called CFTR. Deborah Nelson at the University of Chicago, Illinois, and her colleagues have now shown that this protein helps to control pH in the digestive compartment of macrophages that reside in the lungs. Macrophages kill bacteria and other invading pathogens by engulfing them and drowning them in acid.

The team showed that macrophages in the lungs — but not the abdomen — in CFTR-deficient mice were less able to maintain strong acidity, and therefore less able to fight infection.

MICROBIOLOGY

Toxin factory

J. Am. Chem. Soc. doi:10.1021/ja062953o (2006)

Researchers in Germany have pulled off the seemingly impossible task of isolating and culturing an endosymbiotic bacterium on a large scale.

Christian Hertweck and his colleagues at the Leibniz Institute for Natural Product Research and Infection Biology in Jena grew the bacterium *Burkholderia rhizoxina*, which usually lives symbiotically in the cytoplasm of the fungus *Rhizopus microsporus*.

This bacterium produces a toxin known as rhizoxin, which blocks cell division. It causes

blight in rice seedlings infected by the fungus, but is also a potent anticancer agent.

Their culture yielded high concentrations of rhizoxin, and other chemically related compounds that were up to 10,000 times more potent than rhizoxin as anticancer drugs *in vitro*.

HYDROGEN STORAGE

Quick release

Angew. Chem. Int. Edn doi:10.1002/anie.200601434 (2006)

Hydrogen-fuelled engines would be a boon for the local environment, producing an exhaust that is only water. But we lack a suitable, safe way to store the explosive gas. Andrew Weller at the University of Bath, UK, and his colleagues suggest a new approach.

The team prepared clusters of rhodium atoms that reversibly bind molecules of hydrogen. Unlike other hydrogen-storage systems, the clusters hold the gas at ambient

temperature and pressure. The hydrogen could be released within milliseconds — either by applying a current, or adding a chemical. However, the amount of hydrogen that can be stored in a given volume of material is, for now, below a practical level.

CANCER BIOLOGY

Partners in crime

Cell 126, 489–502 (2006)

A key protein involved in breast cancer has an accomplice, say researchers. The finding helps to explain how the tumours become invasive.

Just under a third of breast cancers contain extra copies of a signalling protein called ErbB2, which promotes cell division. Now Filippo Giancotti of the Memorial Sloan-Kettering Cancer Center in New York and his colleagues have found that another protein, $\beta 4$ integrin, can boost the signal from ErbB2.

The two proteins club together to promote the activity of genes that disrupt cell and tissue structure, and increase cell division. Targeting $\beta 4$ integrin could improve the efficacy of drugs such as Herceptin that block ErbB2 activity, the researchers suggest.

NEUROSCIENCE

Fathering changes brain

Nature Neurosci. doi:10.1038/nn1753 (2006)

It's not clear how it happens, or why — but male marmosets respond to becoming fathers by growing new bits of neuron.

A team led by Elizabeth Gould of Princeton University, New Jersey, compared brain tissue from marmoset fathers to that from adult males living in mating pairs without offspring.



NASA, METI & ERDSAC

Male marmosets carry an infant for as much as 70% of its first month of life.

Both first-time and experienced fathers had a higher density of dendritic spines — which branch out to form connections between neurons — in their prefrontal cortex than did non-fathers. Increased expression of a certain vasopressin receptor hints that the hormone may play a role in this structural change.

MATERIALS SCIENCE

Shrink to fit

Science doi:10.1126/science.1132195 (2006)

An ion wrapped in a crowd of solvent molecules will distort to squeeze into a pore smaller than itself. This, say Yury Gogotsi of Drexel University in Philadelphia, Pennsylvania, and his colleagues, may provide a strategy to optimize energy storage in carbon materials riddled with holes.

Porous carbon materials soaked in electrolytes are used as supercapacitors, which store energy in charged ions adsorbed to the surface. Contrary to expectations, Gogotsi's team found that such a material's capacitance increases suddenly if the pore width is shrunk below the size of the electrolyte ion's solvation shell. The effect is caused by the distortion of the shells, which pushes their enclosed ions closer to the carbon surface, increasing the number of ions per unit area.

EARTH SCIENCES

Satellite maps fault line

Earth Planet. Sci. Lett. doi:10.1016/j.epsl.2006.06.025 (2006)

Researchers have found a way to use readily available satellite photographs to measure the ground deformation caused by large earthquakes.

Geophysicist Jean-Philippe Avouac and his

team at California Institute of Technology in Pasadena, analysed the 7.6-magnitude earthquake that hit Kashmir on 8 October 2005. The team compared before and after images from the Earth-observation satellite Terra. After compensating for distortions caused by the imaging system and the mountainous terrain, they mapped a surface rupture (see graphic above) over 75 kilometres that had an average offset of 4 metres.

Avouac hopes that the new technique, which yields detailed displacement data along an entire fault, can be coupled with on-the-ground measurements to create more realistic models of how earthquakes unfold.

EVOLUTIONARY BIOLOGY

A wrinkly mutant

Nature Genet. doi:10.1038/ng1867 (2006)

A study carried out at Oxford University, UK, by Christopher Knight and his colleagues has mapped how a mutation in a single gene can have knock-on effects in many different proteins. Biologists know that some such effects can be good, others bad — showing how an organism must compromise to adapt to its environment.

The mutation in *Pseudomonas fluorescens* lets the bacteria, which would usually just float around, thrive in wrinkly mats on the

surface of nutrient solution. It also resulted in changes to the levels of 52 proteins, which are functionally related, but were not needed to form the mats. Pathways involving these proteins are already implicated in some of the bad effects of the wrinkly mutation, such as impairment of the bacteria's ability to process some nutrients.

CHEMISTRY

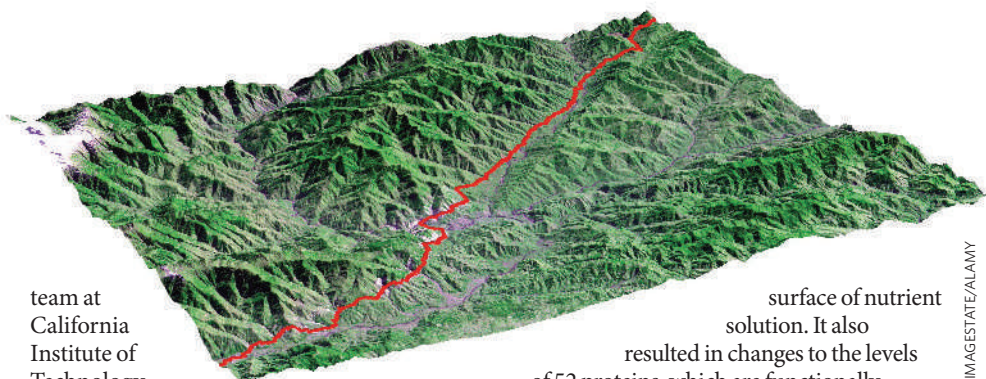
In and out of the mix

Science 313, 958–960 (2006)

Chemists have created an elegant surfactant to stabilize emulsions such as those formed by oil droplets in water. The molecule's surfactant properties can be turned on and off, and it is inexpensive, non-toxic and simple to use.

Designed by Philip Jessop of Queen's University in Ontario, Canada, and his colleagues, the surfactant has a hydrocarbon tail attached to a chemical group known as an amidine. The amidine switches from a neutral, oil-soluble state to a charged, hydrophilic state on reacting with carbon dioxide and water. The molecule then prefers to sit with its tail in an oil droplet and its head in water, stabilizing the emulsion. Bubbling air through the mix returns the amidine to its neutral state, breaking up the emulsion.

Such a surfactant could find application in the oil and polymer industries, which often need to recover material from emulsions formed in an earlier processing stage.



JOURNAL CLUB

Lora Hooper
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A pizza-loving microbiologist is grateful to have her gut microbes on her side.

When I started working in gut biology about 10 years ago, we knew that microbial cells outnumber human cells in the gut by a wide margin. But we had very little good information about

exactly which microbes were there or what they were doing.

So I was delighted when David Relman's group at Stanford University published a paper in which they used state-of-the-art molecular techniques to get a better fix on the microbial make-up of the human gut (P. Eckburg *et al. Science* 308, 1635–1638; 2005). They showed that in the human gut, representatives of three different kingdoms of life (Eucarya, Bacteria and Archaea) coexist.

Karen Nelson's group at The Institute for Genomic Research in

Rockville, Maryland, has recently gone even further to provide insight into exactly why we offer a safe haven to our gut microbes (S. Gill *et al. Science* 312, 1355–1359; 2006).

By analysing the DNA sequences present in the collective genomes of the human gut microbiota, her group revealed that a lot of microbial effort goes into breaking down complex carbohydrates in our diet that we can't digest by ourselves. We humans clearly profit from microbial help in extracting dietary

energy that would otherwise be lost.

Interestingly, there is even evidence of inter-kingdom collaboration between Archaea and Bacteria in determining which polysaccharides are broken down first (B. Samuel & J. Gordon *Proc. Natl Acad. Sci. USA* 103, 10011–10016; 2006).

I always remind myself that my gut microbes are in it for themselves, but I'm glad they're cooperating for my benefit. I just wonder whether they know what to do with my pizza-rich diet.

NEWS

G. ROBINS/AFP/GETTY IMAGES



Light shed on battle against HIV

TORONTO

Why does the human body fail to defend itself from the virus that causes AIDS? The answer, researchers are finding, could be that HIV triggers a natural mechanism that impairs the main cells responsible for fighting the virus.

As a result, these cells, called killer T cells, eventually become exhausted and give up their fight. Now, research indicates that giving these tired cells a boost may be simple, and clinical trials using this strategy could start as early as next year.

"This is really a very important phenomenon, and it explains a lot of things," says Rafick-Pierre S  kaly of the University of Montreal in Quebec, senior author of one of two papers published on 20 August. "We would like to be able to push this into patients and show whether this has an effect."

Clue in mice

The story began last December, when Rafi Ahmed of Emory University in Atlanta, Georgia, reported work on mice infected with a chronic viral infection¹. His group studied the T cells these mice made to target the long-term infection. The researchers found that the cells expressed much higher levels of a protein called PD-1 than mice infected with a short-term infection. This was intriguing, because scientists already knew that PD-1 can act as a

brake on T cells that express it, forcing them to shut down their fight against infections.

Even more tantalizing was Ahmed's finding that he could release the PD-1 brake by injecting the mice with a molecule to block PD-1's interaction with another protein, called PD-1 ligand. That protein triggers the PD-1 molecule to shut off T cells. Stopping the deadly interaction between PD-1 and PD-1 ligand seems to release the restraint on T cells, Ahmed reported.

Researchers then raced to examine PD-1 in people. Teams included one led by S  kaly, now reporting results in *Nature Medicine*², and another led by Bruce Walker of Massachusetts General Hospital in Boston, who reports results in *Nature*³.

Both teams found that, in human patients, the T cells that should be fighting off HIV express very high levels of PD-1. Both papers also show that levels of PD-1 expression correlate with the amount of HIV a patient has in his or her body, indicating that the protein has something to do with how well the patient controls the virus. And both papers report that interfering with the partnership between PD-1 and PD-1 ligand can rejuvenate the cells' fight against the virus.

"Exhaustion has been a proposed mechanism

of T-cell failure in HIV disease for a long time," says Michael Lederman, an immunologist at Case Western Reserve University in Cleveland, Ohio, who was not involved in the work. "What seems nice about this piece is that it explains some observations that have been made for some time, and it may apply to other infections. And there's an ability to intervene."

Future trials

At least one company is already looking at ways to use the finding. Medarex, based in Princeton, New Jersey, is testing a protein that interferes with the PD-1/PD-1 ligand partnership. The company is interested in it as an anticancer agent at the moment, but is also talking to HIV researchers about potential clinical trials.

The finding could also help illuminate other chronic viral diseases, such as hepatitis C. And it could help scientists unravel another interesting story: how certain people

infected with HIV can control their infections naturally, without drugs, and almost never get sick with AIDS.

On 16 August, Walker and S  kaly announced that they are beginning the first large-scale study on the genetic make-up of these people, known as '  lite controllers'. S  kaly adds that

"This work is elegant, carefully done and solid — but we've got to be cautious."

A vigil for past victims ends this year's international AIDS conference in Canada.

PD-1 could shed light on the subject. So far, he has studied 12 elite controllers, and all have normal levels of PD-1 expression — much lower than those found in most HIV patients.

Still, other scientists caution that the PD-1 story cannot explain everything. PD-1 is found on many cells, not just those that target HIV. So interfering with it may have unpredictable consequences, says Rick Koup, an immunologist at the US National Institute of Allergy and Infectious Diseases (NIAID).

Koup is publishing a paper on the PD-1/PD-1 ligand interaction in HIV patients on 5 September, in the *Journal of Experimental Medicine*. His findings indicate that blocking PD-1's interaction with PD-1 ligand may simply prevent T cells from dying, rather than rejuvenating them.

Anthony Fauci, director of the NIAID, agrees that further investigation is needed to follow up the PD-1 findings. "This work is elegant and very carefully done, and from a scientific standpoint it's solid," he says. "But we've got to be careful we don't make the majestic leap to say: now we've solved the issue of unresponsiveness in people infected with HIV. It may turn out to be that way, but we've got to be cautious."

Erika Check

1. Barber, D. L. *et al. Nature* **439**, 682–687 (2006).
2. Trautmann, L. *et al. Nature Med.* advance online publication, doi:10.1038/nm1482 (20 August 2006).
3. Day, C. L. *et al. Nature* advance online publication, doi:10.1038/nature05115 (20 August 2006).



THE WATER CRISIS IS HAPPENING NOW

Severe water scarcity arrives decades earlier than predicted.

www.nature.com/news

China set to make fusion history

The world's first fully superconducting tokamak is soon to produce a discharge of ionized gas or plasma.

If all goes as planned, China's Experimental Advanced Superconducting Tokamak (EAST) project will make its first plasma in the next few weeks.

EAST uses superconducting coils to create a magnetic field that confines plasma inside a doughnut-shaped vessel known as a tokamak. The behaviour of the plasma should shed light on the potential of nuclear fusion as an energy source.

Conventional experimental fusion machines use copper coils, or a combination of copper and superconducting coils, to trap the hot plasma. But copper coils heat up and need to be cooled down regularly, thus limiting operating time. EAST has only superconducting coils so it can be operated continuously.

The US\$25-million machine sets the stage for the multibillion-dollar ITER fusion experiment that is to be built in France; ITER, due to start operations in 2016, is similarly designed to be all-superconducting.

"We'll need new energy resources for a long-term period, and fusion will be one of them," says Peide Weng,

deputy manager and chief engineer of the EAST project at the Institute of Plasma Physics of the Chinese Academy of Sciences. "For commercial use, it should be superconducting because it will need continuous operation."

China approved the machine in 1998, as part of a push towards new energy sources. Construction then began in 2000 in Hefei, in southern China. The 150-member EAST team imported some material and components, but designed and fabricated the bulk of the equipment on its own.

EAST is only one-tenth the volume of Japan's JT-60 tokamak, and one-hundredth the expected volume of ITER. It won't produce fusion power, and is designed to study advanced tokamak physics. The first plasma, created from heated hydrogen gas, will probably last for only a few seconds. Still, "it will be a very important step forward," says Toshihide Tsunematsu, director-general of the Naka Fusion Institute of the Japan Atomic Energy Agency, who visited EAST a few weeks ago. The agency owns the JT-60 tokamak.

Eventually, the EAST team aims to hold a plasma for study for as long as 1,000 seconds. In other tokamaks plasmas last

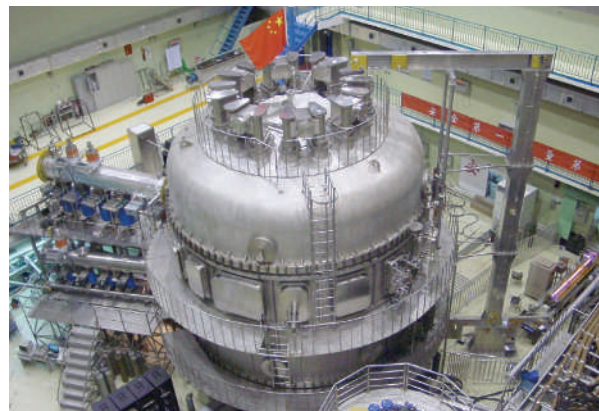
for only a few tens of seconds.

South Korea is currently developing a tokamak similar to EAST, called the Korean Superconducting Tokamak Reactor (KSTAR), whose construction is expected to be completed at the end of 2007. Japan also plans to upgrade its JT-60 machine to make it fully superconducting in a few years.

International physicists praise what China has accomplished so far. In 2003, 25 physicists visited EAST as part of its international advisory committee.

"Everybody came away very impressed," says Dale Meade, a physicist with the Princeton Plasma Physics Laboratory in Princeton, New Jersey, and a member of the group. The committee plans to hold another meeting in October, when China hosts a conference of the International Atomic Energy Agency.

In the meantime, the EAST researchers have plenty to work on, says Tsunematsu. They will have to improve key technologies, such as a device to heat the plasma, and be able to effectively control high-temperature plasma for a long period of time. "China will face a real challenge," he says. ■
Ichiko Fuyuno
With additional reporting by Geoff Brumfiel



In the lead: if China's EAST project is a success, it will pave the way for other major fusion experiments around the world.

INST. PLASMA PHYS., CHINESE ACAD. SCI.

COREIS

Cool blue: increased cloud cover may mean less Sun is getting to the world's oceans.

Oceans cool off in hottest years

Ocean measurements suggest the world's seas have cooled substantially during some of the warmest years in recent history. If real, the dip is likely to reflect a short-term fluctuation in an ocean that is warming overall, say climate scientists.

The years 2003 and 2005 saw the highest global average surface temperatures in more than a century. An upcoming paper in *Geophysical Research Letters* reports that during this period, the upper 750 metres of the oceans lost around one-fifth of the heat accumulated over the past 50 years.

But don't read too much into that, oceanographers warn. "Cooling on a short-term scale doesn't challenge the long-term warming trend," says Sydney Levitus, director of the World Data Center for Oceanography in Silver Spring, Maryland. "What it does tell us is that we still don't sufficiently understand how the global climate system works."

Oceans cover two-thirds of Earth's surface and have far more heat-storing capacity than air or land. Overall, they have warmed in recent decades. Between 1955 and 1998, for example, all of the world's oceans, down to a depth of 3,000 metres, warmed by 0.037 °C (S. Levitus, J. Antonov and T. Boyer *Geophys. Res. Lett.* 32, L02604; 2005).

But that warming has not proceeded steadily. The trend has reversed for a few years at least once during the past half century. In fact, a drop in temperature that occurred between 1980 and 1983 was almost twice as pronounced as the current cooling.

But only for this latest cooling episode have oceanographers obtained good enough global data to provide a convincing argument that the effect is real. The report relies in part on data gathered by ARGO, a global array of 2,500 floats. The ARGO floats descend to various depths to probe ocean temperature and salinity, and surface occasionally to transmit their data via satellites.

When ARGO data were removed from the analysis, the cooling was significantly less pronounced, and the error bars became larger, say the authors of the report, led by John Lyman of the National Oceanic and Atmospheric Administration in Seattle, Washington. Some researchers suspect that ARGO has provided the ability to detect short-term temperature fluctuations. "We may be seeing different things just because we are looking harder," says Brian King, a physical oceanographer at the Southampton Oceanography Centre, UK.

"We may be seeing different things just because we are looking harder."

Better observation systems are showing the oceans are more changeable than some people thought, says Gavin Schmidt, a climate modeller at the NASA Goddard Institute for Space Studies in New York City. A short-term cooling blip can't say much about the climate system in general. But the latest study suggests, he says, that coupled ocean-climate models fail to adequately capture intermittent fluctuations in ocean temperature.

In theory, the recent cooling should have led to a 2-millimetre drop in sea level due to the thermal contraction of water, which becomes denser at lower temperatures. But satellites have measured a steady rise in sea level between 1993 and 2005 — years covered by the study. For the observations to be reconciled, melting ice sheets in Antarctica and Greenland must be contributing far more to sea-level rise than previously suspected, says Schmidt.

Of course, much of the observed ocean-temperature decrease could be an artefact of sampling, says Tim Barnett, a climate researcher at the Scripps Institution of Oceanography in San Diego, California. For example, if warmer water has simply redistributed to areas where fewer ARGO floats exist, the net cooling



Asteroid fly-by eludes study

On 3 July, an asteroid zipped past Earth at a distance of some 400,000 kilometres — slightly farther away than the Moon. In theory, something that close ought to be easy to study. But astronomers have struggled to map the size and shape of the space rock — and now say they know why they found it so difficult.

The rock, dubbed 2004 XP14, is one of more than 800 'near-Earth asteroids' that have been identified in orbits that come perilously close to our planet. This particular rock is unlikely to hit us, but astronomers hoped their observations would help establish how diverse such asteroids are and so better quantify the threat they pose.

But the data obtained by the team proved surprisingly hard to analyse. "The asteroid rotates slowly, so its appearance in the images, due to rotation, hardly changed at all," says Lance Benner, an astronomer at NASA's Jet Propulsion Laboratory in Pasadena, California, who led the effort to image the asteroid.

That's unusual. "Most near-Earth asteroids are very fast rotators," says Vishnu Reddy, a graduate student at the University of North Dakota who also observed the object.

Benner and his colleagues imaged 2004 XP14 using a 70-metre radio antenna at the Goldstone Complex in California. At 260 metres across, the asteroid was a lot smaller than earlier predictions of up to 880 metres. This, together with its rotation rate of roughly one turn every 500 hours, meant that the images the team received barely changed during recording sessions of up to 2 hours. As a result, the researchers could not build the detailed picture of the rock that they wanted.

Earth may be safe from 2004 XP14, but there are plenty more asteroids out there that might collide with us. Having identified three-quarters of the candidates 1 kilometre or more in diameter, NASA plans to widen its search to include objects as small as 150 metres across. And in July, the International Astronomical Union formed a committee to keep it up to date about asteroids that may pose a serious threat to our planet.

Heidi Ledford

could be smaller than the data suggest.

But assuming the trend is real, what caused the cooling and where did the heat go? Aerosols, volcanic activity and small changes in ocean circulations and convection processes may all play a role, says King. But the simplest explanation is that less sunlight has reached the ocean surface as cloud coverage has increased.

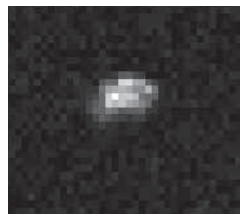
Global cloud coverage has increased by 1–2% since 1999 according to data from the International Satellite Cloud Climatology Project, presumably because of global warming and increased evaporation. Other factors — such as cloud albedo (the extent to which cloud reflects solar radiation) or cloud-top temperature — might have changed in the same period, says William Rossow, a cloud expert at the Goddard Institute. "It is completely insufficient to look for a simple explanation of a short-term temperature change," he says, "as the climate system is much more complex."

The heat itself could be hidden at greater depths in the ocean, or — more likely, says Schmidt — it may have escaped into the atmosphere and out into space. Yet a corresponding change in Earth's radiation budget has not been observed.

"The system's internal variability might well be larger than we thought," says Levitus. "This is exciting news, but food for global-warming sceptics it is not."

Quirin Schiermeier

L. BENNER, JET PROPULSION LAB.



Blip on the horizon: radar imaging of the asteroid 2004 XP14 proved to be more difficult than expected.

SPECIAL REPORT

What happens after the water recedes?

New Orleans universities are still struggling to recover from the ravages of Hurricane Katrina.

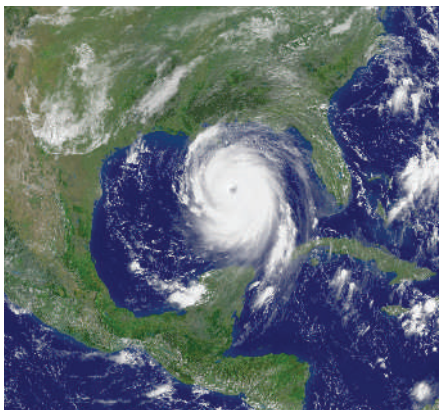
Heidi Ledford examines how researchers at one leading institution are coping.

On a grim day last September, a ten-car convoy of researchers drove into the putrid waters of downtown New Orleans. Scientists fanned out across the Tulane University health-sciences campus, salvaging computers and research samples from buildings flooded in the aftermath of Hurricane Katrina.

Today, the university campus buzzes with activity of a different sort. Undergraduate classes resumed in January, with 93% of students returning for the spring semester. Research experiments are again under way. And preliminary numbers suggest that Tulane earned \$133 million in sponsored research last year — a mere 3% drop from the previous year.

Tulane has emerged relatively unscathed compared with other institutions in New Orleans (see 'Around New Orleans', opposite). Yet even at Tulane, Katrina has left a permanent mark in the form of low morale, lost equipment and laid-off faculty members.

On 29 August 2005, Katrina separated Tulane's two campuses by more than just geography. Floodwaters barely reached the stately buildings of the university's historic uptown campus for undergraduates. Science departments there were up and running after a few rough months of limited electricity and minor



As Hurricane Katrina headed for New Orleans, few were anticipating the flood it left in its wake.

repairs. "On the undergraduate campus, you'd be hard pressed to find any evidence that there was a storm," says Robert Garry of the department of microbiology and immunology. "But other parts... well, it's not so good."

These other parts of Tulane are located downtown, nearly 5 kilometres away, on the health-sciences campus. There, the waters rose to 2 to 3 metres, flooding electricity supplies and maintenance rooms on the lower floors. Some research labs began operating again as early as November, but others didn't

reopen until January (see 'Winds of change: three stories from Tulane's faculty', below). Even then conditions were difficult: in the J. Bennett Johnston Health and Environmental Research Building, the first health-sciences building to reopen, some researchers were left warming their hands over Bunsen burners when temperature controls failed last winter.

Back in August last year, electricity losses also doomed many experiments. It is safe to say that no one working in the biomedical sciences at Tulane will ever again take refrigeration for granted. Power failures and warm temperatures destroyed stem-cell lines, tissue cultures and antibodies, some of which were the products of years of specialized work. Only some samples were saved through emergency rescue efforts.

"We lost in excess of \$100 million of biological assets," says John Clements, dean of research at the medical school. "But we also saved \$100 million of biological assets." Researchers credit the National Institutes of Health and the National Science Foundation with providing resources for recouping their losses, and Tulane itself has dedicated \$20 million to research grants to be awarded over the next two years.

Others lost far more — their jobs. The university, having sustained property damage

Winds of change: three stories from Tulane's faculty

Tyler Curiel: Leaving

After the storm, the flood, helicopter rescues amid gunfire and a four-month exile in Colorado, cancer researcher Tyler Curiel has made a decision he says is "very painful". He is about to leave New Orleans for good.

In September, he will become the director of the University of Texas Health Science Center in San Antonio. Curiel says that in New Orleans, the poor conditions of his lab facilities and of the rest of the city are hindering his research too much. His Tulane lab



still lacks emergency power for its freezers, and he can't find enough candidates to take part in clinical trials because the university hospital has so few patients.

"What worked before Katrina does not necessarily work post-Katrina," Curiel says. "And my kind of research just does not work here."

Prescott Deininger: Staying

The associate director of Tulane's cancer centre knows he was lucky. His house did not flood. His lab was one of the first at the university



to reopen. His lab members all returned. Now Prescott Deininger is back to writing papers and applying for grants.

But his job has changed a little. He has had to go from lab to lab talking to investigators to find out what it will take to convince them to stay at Tulane. It's hard for him not to take it personally when faculty members leave. "It tears the guts out of me," he says.

Cesar Fermin: Recovering

In December 2005, Cesar Fermin was a tenured faculty member at the Tulane medical school, studying hair cells of the inner ear. The next month, he was a handyman at Fermin's

HandyWorks, repairing damaged homes.

After 15 years as a professor of pathology, Fermin was laid off in December. Unable to leave New Orleans for financial reasons, he decided to pick up the tools in his garage to earn a living.

Fermin soon began to miss the intellectual challenge of academia. "When you're going to put 40 sheets of Sheetrock [plasterboard] on a house," he says, "you start on Monday and on Wednesday you're still hanging Sheetrock." He is applying for academic positions outside New Orleans. **H.L.**





INTERNATIONAL ASTRONOMICAL UNION
Check our website for news as astronomers debate the definition of a 'planet'.
www.nature.com/news

G. BACON/NASA/ESA
D. PHILLIP/EMPICS/AP



More than a week after the flooding began, Tulane University's athletics track was still under water.

of \$160 million, sacked 180 medical-school faculty members on 8 December. Another 50 faculty members were laid off uptown, and the departments of mechanical engineering, civil engineering and computer science were eliminated altogether. Even today, the firings are perhaps the sorest spot for Tulane faculty members, with many questioning the decisions.

Prescott Deininger, associate director of Tulane's cancer centre, says that the disaster demanded drastic action. He adds that he was consulted before the lay-offs were finalized, and he feels that his recommendations were taken into consideration before final decisions were made by the president's office and approved by the university's board of administrators.

Tyler Curiel, chief of haematology and medical oncology, agrees that the situation was dire but says that the process of deciding which jobs to cut was not transparent enough. Although

he was consulted early in the lay-off discussions, he says that he was not involved in any final decisions, nor was he ever clearly told how decisions were to be made. Meanwhile, he was fielding frantic phone calls from faculty members asking if they were going to be 'let go'.

"It was a very frustrating time for all of us," says Curiel, who is leaving Tulane this month for the University of Texas Health Science

Center in San Antonio. "It just wasn't handled well from a human standpoint."

The American Association of University Professors (AAUP) has been investigating the firing decisions at Tulane. Jordan Kurland, the association's associate general-secretary, says that

Tulane mostly abided by the AAUP's recommended standards, which allow tenure to be cancelled in cases of extreme financial need. But more could have been done, he suggests. "Tulane authorities have provided general information as to the financial health of Tulane," says Kurland. "But they have not, despite urg-

"If you're on a ship and the ship is sinking, the captain doesn't want to poll the deckhands to figure out what to do."

ings to do so, taken the next step and explained why they fired these particular people."

Clements says that critics don't understand the magnitude of the crisis. "If you're on a ship and the ship is sinking, the captain doesn't want to poll the deckhands to figure out what to do," he says. He also notes that Tulane has offered its laid-off clinical faculty members a one-year severance package — which amounts to a better deal than those provided by other institutions in New Orleans.

Including lay-offs and voluntary resignations, Tulane University has lost 17% of its full-time faculty members in the past year. Many of the remaining faculty members have been approached by other universities.

Administrators hope that the faculty exodus is now over and that those who have stayed this long are committed to staying. But Curiel predicts that there may be another wave of resignations on the horizon. Pharmacologist Bruce Bunnell says he couldn't help but entertain the thought of leaving. "Tulane's been fantastic to me, but science is one of these things where you make your own way," he says. ■

Around New Orleans

Tulane University fared better than most academic institutions in the city.

Louisiana State University

The medical school started up again at the university's Health Sciences Center in January, despite damages worth some \$200 million. All but 1 of 22 campus buildings will be at least partially reopened by this autumn. The centre lost 170 faculty members to resignations, retirement and temporary lay-offs. An additional 1,900 staff members were also laid off.

University of New Orleans

Nineteen faculty members in the sciences have resigned since last autumn, and numerous others retired or were temporarily laid off. The loss is expected to cost the university about 40% of its revenue from scientific research grants.

Loyola University New Orleans

In early August, Loyola sued its main property insurer for more than \$20 million in unpaid Katrina losses. Projected freshman enrolment for this autumn is down about 40%.

Xavier University of Louisiana

Post-Katrina flooding submerged Xavier's campus under more than 2 metres of water, and the university says it needs to raise \$50 million to cover the damages. Autumn undergraduate enrolment is down about 40%.

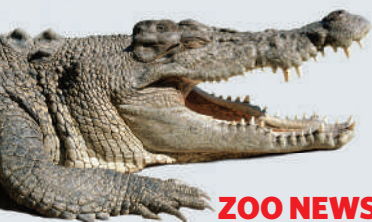
ON THE RECORD

“We are seeking a jury of twelve — the most qualified and the most cynical.”

Engineers at Irish company Steorn are so convinced that they have developed a source of free energy, they are challenging scientists worldwide to prove them wrong.

“They’re pretty small. Maybe we can bring in a ping-pong ball.”

Geza Gyuk on adding new planets to Chicago’s Adler Planetarium.



ZOO NEWS

Snap decision

As astronomers discuss the definition of planets, the Australian government has been considering what creatures to describe as fish. In a law meant to aid regulation of aquatic commerce, passed on 17 August, some were surprised to see that crocodiles made the grade, alongside prawns and scallops.

Preposterous piglets

Officials at the Copenhagen Zoo in Denmark were stunned last week when they discovered five piglets in the den of a babirusa, a wild hog native to Indonesia. The newborns apparently resulted from the animal’s tryst with a domestic pig who kept it company in its cage. Zoologists say the two species are only remotely related, and the mating is equivalent to a cow and a goat producing offspring.

NUMBER CRUNCH

The ozone hole is recovering, but not very quickly...

29 million km² was the maximum size of the Antarctic ozone hole in 2000 — the biggest ever.

27 million km² was the maximum size of the ozone hole five years later.

59 years is how long it should take for the hole to close.

Sources: AP, World Meteorological Organization

Early embryos can yield stem cells... and survive

A single cell can be teased from a human embryo and used to produce stem cells while leaving the embryo intact. The process, published online in *Nature* this week, could enable stem-cell lines to be generated without the controversial destruction of human embryos — but some ethical objections remain.

Embryonic stem cells, prized for their ability to make other tissue types, are typically extracted from an embryo that has developed into a hollow ball called a blastocyst. The process pulls the embryo apart and destroys it.

This week’s paper shows that stem-cell lines can be grown from less developed embryos — balls of eight to ten cells — and the process could leave them unscarred (I. Klimanskaya *et al. Nature* doi:10.1038/nature05142; 2006).

The technique is similar to that used for pre-implantation genetic diagnosis, an option during *in vitro* fertilization (IVF) in which a single cell is extracted from an embryo and tested for genetic disorders. Last year, a team led by Robert Lanza of Advanced Cell Technology in Worcester, Massachusetts, showed that single cells extracted from mouse embryos in this way could be grown into stem-cell lines (Y. Chung *et al. Nature* **439**, 216–219; 2006).

Since then the team has taken cells from 16 spare IVF human embryos, and put them into culture. From a total of 91 cells, the researchers grew two embryonic stem-cell lines that have survived for eight months so far and are able to form different types of tissue. In the experiment, the embryos were dismantled cell by cell; but other embryos should survive the extraction of a single cell, just as they do in pre-implantation genetic diagnosis. Lanza says that the researchers should be able to achieve a higher success rate for cell lines by adjusting the cell

culture conditions. Several other groups have been trying similar approaches, but with no reported success so far.

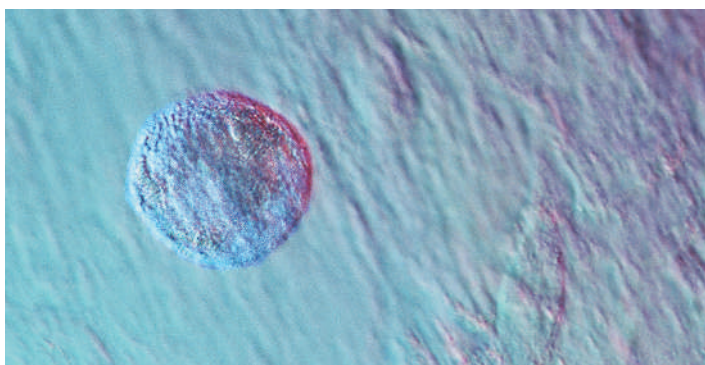
“It shows it’s possible to make any number of lines in future without harming embryos or impairing their development,” says bioethicist Ronald Green, head of Dartmouth College Ethics Institute in Hanover, New Hampshire, and an ethics adviser to Lanza’s company. “I think it’s a way out of the moral impasse in the United States.” President George W. Bush has limited federally funded research on human embryonic stem cells to lines derived before August 2001, on the grounds that he is opposed to destroying embryos to create more lines.

Still, the technique is unlikely to answer all ethical concerns (see *Nature* **437**, 1076–1077; 2005). There are fears that removing a cell from an embryo will lower its chances of implantation in the womb, or alter its development and cause later health problems for the resulting child. Lanza answers this by saying that the risks of the procedure are minimal and that it would only be performed on embryos that are to undergo pre-implantation genetic diagnosis anyway. Others object that the removed cell itself may have the potential to develop into an entire new embryo, and that this is being destroyed.

The method joins a raft of other techniques that have been proposed for deriving ethically sound human embryonic stem cells, such as using embryos that have been genetically altered so that they cannot develop into babies. “None of those methods was likely to satisfy all the critics, and I don’t think this one will either,” says Tom Murray, president of the Hastings Center, a bioethics research institute in Garrison, New York.

Helen Pearson

V. STEGER/SPL



Human embryonic stem cells can develop to form any kind of tissue, and could be used to treat fatal diseases.

Disgraced Korean cloner claims to be back in the lab

Woo Suk Hwang, the South Korean cloning researcher who is currently standing trial, has begun research again (see *Nature* 442, 121; 2006). According to his lawyer, Hwang started work on pig-cloning projects earlier this month.

Hwang has previously claimed to have cloned pigs and has said he hopes to genetically engineer the animals to provide organs for human transplantation. His claims have been questioned since he was shown last year to have published fraudulent papers on human cloning experiments. His group also created the first cloned dog, a finding that has held up to scrutiny.

Hwang is on trial on charges of embezzlement, fraud and violation of a South Korean bioethics law. Hwang's lawyer, Geon Haeng Lee, denied reports that Hwang's research initiative was a publicity stunt designed to affect the trial. "The first time a newspaper tried to run the story, I begged them not to, but they did it anyway."

National Cancer Institute appoints next director

John Niederhuber will be the next director of the US National Cancer Institute (NCI), the biggest component of the National Institutes of Health. The 15 August announcement from President George W. Bush had been widely expected, as Niederhuber has been acting director of the NCI since June.

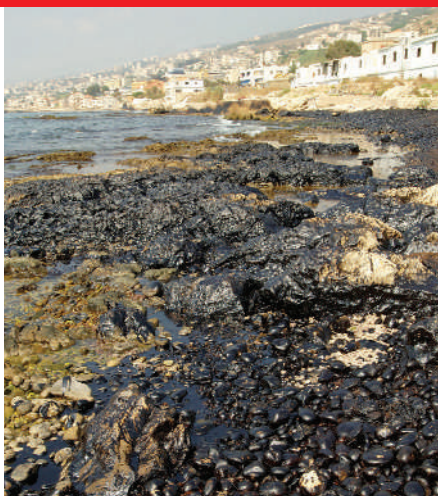
Niederhuber once led the University of Wisconsin Comprehensive Cancer Center in Madison and is an experienced cancer surgeon. He plans to continue running a lab at the NCI in Bethesda, Maryland.

Cancer researcher Mary-Claire King, of the University of Washington in Seattle, is pleased with the choice: "I knew Niederhuber long ago when he was at Stanford. He was extremely intellectually supportive."

Clean up of Lebanon oil spill starts as guns fall silent

The ceasefire between Israel and Hezbollah on 14 August has allowed work to start on a plan to clean up the oil slick created by Israeli bombing of a coastal power plant in Lebanon more than a month ago. About 15,000 tonnes of oil are believed to have spilled into the Mediterranean Sea from the Jiyeh plant, creating a slick affecting some 150 kilometres of Lebanese and Syrian coastline (see *Nature* 442, 609; 2006).

Representatives from the United Nations Environment Programme and the



Slick operation: an oil spill off the Lebanese coast can be cleaned up while the ceasefire lasts.

International Maritime Organization met in Athens on 17 August and backed a €50-million (US\$64-million) clean-up plan. The first task is an aerial survey to verify where the oil lies. Recovery of oil from high-priority sites, including ports and fisheries, will start soon. In the longer term, important habitats for birds and turtles will be protected. Experts from the European Commission and the Regional Marine Pollution Emergency Response Centre for the Mediterranean Sea are already working in the area.

Three quit NASA science committee over budget cut

Three members of NASA's science advisory committee have resigned, two of them after being asked to leave by the agency's administrator, Michael Griffin. The departures are the latest fallout from NASA's decision to cut space science to fund plans to explore the Moon and Mars.

Harrison Schmitt, who chairs the advisory council that oversees the committees, says the science committee had some difficulty coming to grips with the agency's

new mandate. "I think they struggled to recognize that it's a whole new ballgame," Schmitt says. Earlier this year, scientists, including some on the council, spoke out against a NASA proposal to cut space science by 17% between 2007 and 2010 (see *Nature* 439, 768–769; 2006).

The departing scientists are Wesley Huntress, director of the Geophysical Laboratory of the Carnegie Institution of Washington; Eugene Levy, a physicist and provost of Rice University in Houston, Texas; and the committee's chair, Charles Kennel, director of the Scripps Institution of Oceanography in San Diego.

Indian scientists up in arms over nuclear deal

Eight leading Indian nuclear scientists have protested against a planned nuclear deal with the United States (see *Nature* 436, 446–447; 2005), saying it would compromise research and prevent the development of nuclear weapons. The deal is intended to provide access to cheaper fuel and technical knowhow.

The group, which includes P. K. Iyengar and two other former chairmen of the Indian Atomic Energy Commission, complained in a 15 August memo to Indian parliamentarians. The scientists say that centres such as the Tata Institute of Fundamental Research in Mumbai would become subject to US scrutiny, and call for restrictions proposed under the deal to apply only to facilities using imported nuclear material. They say plans to separate military and civilian nuclear research would also undermine India's weapons programmes.

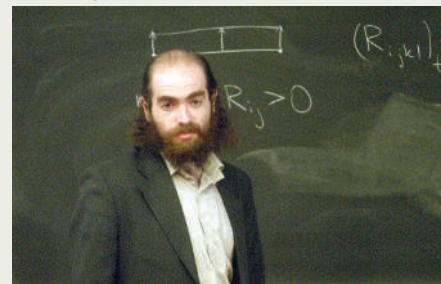
Prime Minister Manmohan Singh later said India would never compromise on its nuclear weapons programme and that the final deal would not allow external supervision of research facilities. He has invited the scientists to a meeting on 26 August to discuss their fears.

Russian recluse shuns maths' highest honour

A mathematician widely agreed to have derived a proof of the century-old Poincaré conjecture has declined to accept one of the 2006 Fields medals. The medals, awarded every four years to mathematicians no older than 40, are the most prestigious prizes in mathematics.

Grigory Perelman (right) posted three papers online in 2002 and 2003. They sketched out a proof of the conjecture, which concerns the shape of three-dimensional spaces. He used a tool known as Ricci flow to develop ways of studying the deformation of surfaces (see *Nature* 442, 490–491; 2006). Perelman has since retreated from the public eye. He was not present when the 2006 medal winners were announced on 22 August, but prize organizers said he had "declined to accept" the award.

The other Fields medals went to Andrei Okounkov of Princeton University, Terence Tao of the University of California, Los Angeles, and Wendelin Werner of the University of Paris-Sud in Orsay.



BUSINESS

Getting the right mix

Biotechnology companies come in many shapes and sizes, but, say two economists, the ones with the widest spread of skills in their teams stand the best chances of success. **Aaron Bouchie** reports.

For decades, founders of biotechnology companies have sought a winning formula for their enterprises. The key to success has proven elusive, however. Capable management and good relations with early investors are usually considered essential. But, according to one study, the factor that correlates most closely with a company's success is the diversity of skills encompassed by its research team.

In an unpublished paper called "Knowledge Bridging by Biotechnology Start-ups", David Hsu of the Wharton School of the University of Pennsylvania in Philadelphia and Kwanghui Lim, now at the Melbourne Business School in Australia, examined whether using expertise from disparate scientific fields would help a biotechnology firm succeed.

The authors show that by hiring researchers from various scientific disciplines (for example, experimental genetics and information technology), a company increases its chances to complete an initial public offering and have a drug approved by the US Food and Drug Administration. Scott Stern of Northwestern University's Kellogg School of Management in Evanston, Illinois, says the paper builds on the notion that combining ideas from different disciplines is more likely to yield revolutionary inventions. "A given idea can have a greater impact beyond its initial field. These guys show this elegantly," says Stern.

Lim and Hsu came to their conclusion after looking at the performance over two decades of 19 companies that built their businesses on a set of patents for recombinant DNA technology. The firms ranged from agricultural biotech companies such as Mycogen of Indianapolis and DNA Plant Technology of Oakland, California, to the two largest biopharmaceutical companies in the United States, Amgen and Genentech.

They looked at patent data to determine how many different scientific disciplines were built upon for each technological innovation; inventors are required to cite existing technologies that their patents build on in the same way that research journals require authors to include references. The authors found that the more scien-

tific fields referenced by a company's patents, the more likely the company was to succeed.

And they found that hiring a diverse group of researchers during the early stages of a company's development contributed most to its ability to "use ideas from one technical domain to innovate in another area". Surprisingly, obtaining venture capital backing or forging collaborations with other firms had little effect on the tendency to innovate.

Teamwork

That somewhat defies conventional wisdom: venture-capital deals are supposed to provide access to networks of people that can assist young companies. And partnerships with other firms ought to have a similar effect. Hsu and Lim suggest that venture-capital backing can actually damage innovation by encouraging a focus on short-term success, whereas alliances with outside companies are often established to access marketing or manufacturing capabilities, not new knowledge.

Their study, which is currently undergoing peer review at *Organization Science*, a management journal, is part of a broader effort by economists to use empirical methods to study factors behind the success or failure of young companies. For example, Toby Stuart and his colleagues showed in a 1999 paper (T. Stuart, H. Hoang and R. Hybels *Admin. Sci. Quart.* 44, 315–349; 1999), that a private biotech that has associations

with high-status investors and collaborators was more likely to complete a public stock offering. Lee Fleming of Harvard Business School has been looking at the effect of agreements that restrict the rights of biotech employees to go and work for other firms: he thinks such contracts are stifling the biotechnology industry.

The authors believe their study will help biotech executives modify how they budget their research dollars — although private investors may give such work short shrift. "Most of us investors read these types of study and gain some insight, but they're not driving our decisions," says Steve Burrill, chief executive of Burrill & Company, a San



David Hsu thinks combining skills can fuel innovation.



Francisco-based merchant bank that specializes in biotechnology. He thinks the most important single corollary for success in a young biotechnology company is the quality of its management. "It's better to have mediocre science and a balanced team than one Nobel prizewinner with no support."

Other economists say that diversity of talent came out as important in this particular study because the technology it looked at — recombinant DNA — could end up being used in many different ways. Fiona Murray of the Massachusetts Institute of Technology (MIT) in Cambridge says that if they had looked at companies that specialize in a particular disease, for example, then diversity of staff expertise might have mattered less.

Murray also contends that the best way to understand how knowledge gets passed between researchers is not to do just a patent analysis, but also to interview the researchers and find out directly. "Many folks in industry read patents and build on the knowledge they contain," she says. "But most researchers hear about technological advancements from research papers or



GETTY IMAGES

directly from colleagues.” Hsu and Lim won’t have picked this up, she says, because their analysis relied solely on the patent record to show how innovations were conceived.

Although Hsu acknowledges such limitations, he says there is currently no easy method for adding qualitative data. “We looked at 19 firms over 20 years and we looked at changes within each firm year-by-year,” he says. “How would each inventor remember whom they talked to 15 years ago, 14 years ago, and so on?”

Robert Langer, an MIT chemist with some 500 patents to his name and a long track record of interdisciplinary research, says that having lots of different disciplines in a biotechnology company’s research team is not always necessary for it to bear fruit. “Some research groups might be better off staying within a single discipline,” he says, adding that the most important thing is the mix of personalities in the lab. “It is important to encourage people to reach for their ideas, and create an environment where people can do so,” says Langer. “Having an interdisciplinary team might contribute to that.” ■

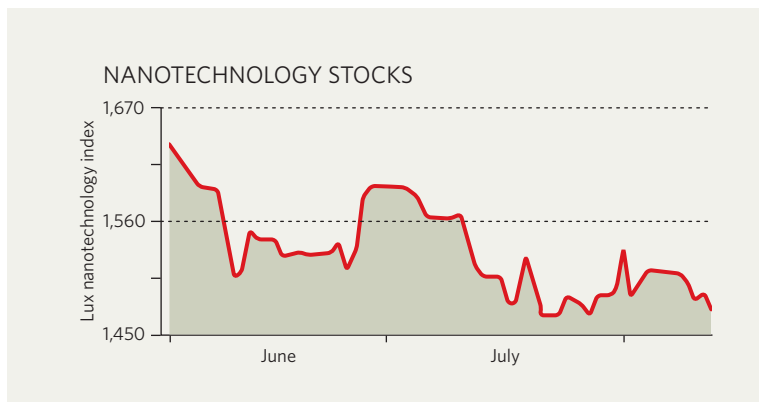
IN BRIEF

COTTON SWEET Monsanto, the world’s leading supplier of genetically modified crop seed, has agreed to buy Delta Pine and Land, a Mississippi-based cotton-seed supplier, for \$1.5 billion. The purchase, announced on 15 August, should end fierce litigation between the two companies over previous efforts by Monsanto, based in St Louis, Minnesota, to take over the Mississippi company. If regulators and shareholders approve the deal, it will widen Monsanto’s commanding lead in the transgenic crop market, where Switzerland’s Syngenta is its main remaining rival.

SUMMER CLEAR-OUT Jeff Kindler, Pfizer’s freshly appointed chief executive, has moved swiftly to reshape top management at the world’s largest drug company (see *Nature* 442, 734; 2006). Karen Katen, one of his rivals for the highest position and head of the company’s pharmacological division, will leave the company altogether, whereas David Shedlarz, the other contender and Pfizer’s finance chief, gains wider responsibilities. Kindler also named a seven-strong taskforce, including research chief John LaMattina, which, he said, would streamline company operations and accelerate decision-making.

CHIP FLOAT German semiconductor-maker Infineon Technologies managed to float its memory-chip arm, Qimonda, on the New York Stock Exchange. The public offering raised just \$546 million — not bad by current standards, but less than half what analysts had predicted when the float was first announced. The offer was hurt by bad market sentiment towards such offerings in all technology sectors and by falling prices for Qimonda’s main product, the memory chips used in personal computers. Some market watchers expressed relief, however, that the float actually went ahead in current market conditions.

MARKET WATCH



Technology stocks are in the doldrums this summer after losses in the spring, and nanotechnology is no exception to the pattern. But within the basket of stocks that makes up the Lux Research index, individual company performance has been mixed.

One undoubted success has been NVE, a Minnesota-based spintronics company, whose stock soared from \$14 to almost \$22 in July on unconfirmed reports that its technology may be successfully incorporated in non-volatile memory chips made by Freescale Semiconductor.

Nucryst Pharmaceuticals, the Massachusetts-based company whose silver nanocrystals are now popular as wound treatments, was also on the up in July after declaring strong sales growth in the second quarter. And according to Peter Hebert, president of the New

York consultancy that runs the Lux index, investors are optimistic that the nanocrystals will eventually prove useful as drug treatments for infections and inflammation.

Less stellar performances were delivered by Cambridge Display Technology, the UK light-emitting diode (LED) display maker, whose shares sank from \$8 to \$5 in July in anticipation of weaker-than-expected sales in the third quarter. Similar concerns dragged down Oregon-based FEL, which makes scanning electron microscopes.

Hebert says an emerging feature of the nascent sector is the prevalence and importance of litigation over its intellectual property. Elan’s July decision to sue Abraxis Bioscience over its use of a nanoparticle formulation technique is, he says, “a sign of things to come”. ■

Colin Macilwain

Make anything, anywhere

Can everyone use technology creatively? Engineers at the Massachusetts Institute of Technology think so and have launched 'Fab Labs' around the world to prove it. **Apoorva Mandavilli** reports.

Neil Gershenfeld has been teaching a class called "How to make (almost) anything" to some of the brightest young adults in the United States for years. But it took an eight-year-old girl in a small village in Ghana to show that anyone, anywhere, really can make just about anything.

One evening in June 2004 — the day after Gershenfeld had left Ghana having taught a week-long version of his class in the village — little Valentina Kwofie began cobbling together a circuit board.

Hours passed. Several times Kwofie's parents stopped by the lab Gershenfeld had set up, imploring, "Valentina, let's go home, let's have dinner, let's go to bed." It was the first time anyone in the village, Takoradi, had made a circuit board: people crowded around, watching her nimble fingers manoeuvre parts half the size of a grain of rice. Finally, long past her bedtime, she crafted a board that worked.

Gershenfeld, director of the Center for Bits and Atoms at the Massachusetts Institute of Technology (MIT), hadn't known what to expect when he put the fabrication laboratory, or 'Fab Lab', technology that he worked with at MIT into the context of rural Africa. What he got was inspiration. "This eight-year-old girl in Ghana was making microcontroller circuit boards for the love of it, for the joy of discovery," he recalls. "That ordinary people can do it and want to do it was very surprising."

The Fab Lab in which Kwofie made her circuit has been followed by others around

the world, each equipped with the same key machines. Today, there are ten Fab Labs — above the Arctic Circle in Norway, in Costa Rica, India and South Africa — and within the next year, fifteen others are planned, of which five will be in Africa.

Together, these labs are showing that giving people the ability to make things for themselves can be the fastest way to solve their problems, particularly in communities with little access to education or technology.

It began in Boston

Gershenfeld began teaching "How to make (almost) anything" at MIT in 1998 as part of an outreach requirement attached to his centre's grant from the US National Science Foundation (NSF). It quickly became the most popular class he taught. Students with no prior technical skills, he says, created the "most wildly expressive things": a web browser for bored parrots, for instance; a container for screams; even a 'defensible dress' that sprouts wires when someone invades its wearer's personal space.

Watching the students' passion for teaching others what they had learned, Gershenfeld toyed with the idea of starting a lab somewhere that was far removed from MIT in its expectations about technology. So he opened a Fab Lab in working-class South Boston. Heartened by

this experience, he decided to start going far afield geographically as well.

He put together a collection of industrial machines, including a laser cutter that makes two-dimensional and three-dimensional objects from non-metal materials, a vinyl cutter that can make flexible antennas, among other things, and a high-resolution milling machine for circuit boards. All are run by simple computers using open-source software written by MIT scientists. This kit today costs about

\$25,000; materials cost another \$10,000. The first labs, like the class that inspired them, were funded by the NSF grant.

"Technical empowerment, problem-solving, invention — that wasn't at all the agenda," he says of the first few labs. "At my end, it wasn't much deeper than curiosity about what would happen."

One of the things that happened was that each new lab took on a character of its own. Whereas the MIT students made fanciful gadgets, many of the developing-world communities seized the opportunity to implement practical ideas they already had but had not been able to realize.

In Ghana, for example, villagers built large 'collectors' to harness solar energy and made machines to grind cassava into fufu powder. In Pabal, India, farmers created sensors, based on a design developed by MIT staff, to meas-

"This eight-year-old girl was making microcontroller circuit boards for the love of it, for the joy of discovery."

— Neil Gershenfeld



Fabulous stuff: the 'fabrication laboratories' have been set up in places as far apart as Maharashtra in India (left) and Lyngen in Norway.



Valentina Kwofie, electronics pioneer.

ure the fat content in milk and help them get a fair price. Farmers in Norway created wireless radios to help them track sheep.

Some labs are on the verge of spinning off small businesses. “Fabs are now moving from experiments to becoming serious tools in addressing developing nations’ problems,” says Sushil Borde, executive director of the non-profit Fab Foundation, who has helped launch Fab Labs in both India and South Africa.

“I really feel that the deepest form of aid that you can give a country is to allow them to design and make their own things,” says Hod Lipson, a mechanical aerospace engineer at Cornell University in Ithaca, New York. “You’re letting them choose what to make. That’s an amazing empowerment.”

A machine that can make anything

Lipson and Gershenfeld are collaborating on the Fab@home project. Their aim is to create a cheaper and more accessible version of existing Fab Labs by scaling things down to just one machine that can make all sorts of things. This machine will fill space with droplets of matter as an inkjet printer fills a page with droplets of colour: a Fab Lab-in-a-box.

This sort of equipment, mostly used for prototyping, is not new and has become steadily cheaper. Most versions work with only one material, however, so Lipson and others are working on printers that can make complex objects from an array of polymers. They have already made simple items, including a battery, from five different materials.

The Fab Lab in Pretoria, South Africa, is the first to have one of the single-polymer ‘3D printers’, but the goal is to make more of them for other Fab Labs, for schools and homes. “One of the nice things about this printer is that you can make it entirely in a Fab Lab, so it’s really scalable,” notes Lipson — although a printer alone couldn’t make another printer.

The self-replicating Fab Lab has yet to materialize, but Gershenfeld and Lipson dream of the day such personal fabricators are as common as a personal computer. Others are not so sure. “It’s hard to believe that everybody would want one,” says Paul Wright, chief scientist at the Center for Information Technol-

ogy Research in the Interests of Society at the University of California, Berkeley.

Wright teaches a class similar to Gershenfeld’s, and his students too have been creative — making, among other things, a pillow alarm clock that can rouse the sleeper without waking a partner or roommate. But, says Wright, “not everyone wants to build things. One has to be realistic.” More likely, he says, is that high schools or community groups would want personal fabricators.

From Norway to South Africa

MIT fields almost daily requests to build new Fab Labs. The ones that make the cut are inevitably driven by passionate local champions. The Norway lab, for example, is led by Haakon Karlsen, a charismatic chief herder, who helped it grow from a room in a barn to a beautiful 11-building Fab Village that has had 4,000 visitors since it opened last year. The village has support from the Norwegian government and local industrialists, and is set to launch a few local businesses.

One of the newest labs is in Soshanguve, a poor township about 45 kilometres north of Pretoria, and is run by a local group, the Bright Youth Council. When the MIT researchers arrived in Pretoria in September 2005, they found that the Soshanguve group already had its own printing centre to help community members with their resumés. The group had pooled money to buy a computer and those who knew how to use it taught everyone else.

Convinced by the council’s enthusiasm, Amy Sun, a graduate student at MIT, set up a Fab Lab between the printing centre and the health clinic with support from South Africa’s Department of Science and Technology. Many who come to the clinic stop by out of curiosity.

When I visited the lab on a warm afternoon in mid-February, just two weeks after it had opened, it was humming with nearly 20 people. About a dozen Youth Council members, with an average age of 25, work there. Because of the demand, the day is organized into two-hour slots allotted to different age groups — and that doesn’t mean just

boys and men. One participant was a pregnant 18-year-old high-school dropout. Another was a 40-year-old housewife who had never seen a computer before the lab opened but is now using one to design decorative cutout designs.

People at the Soshanguve lab have made a light switch controlled by a cellphone, a motion-sensor light and an alarm system — useful devices in the many unsafe neighbourhoods in the area. Some choices are surprising. “There

“The deepest form of aid you can give a country is to allow them to design and make things.”

— Hod Lipson

are boys there at 1 a.m. stringing beads cut on the laser cutter and really liking it," Sun says.

A lab can be up and running within a week of delivery, but the tough part is helping people make things. Sun spends much of her time teaching physics and electronics to people with no knowledge of either who don't speak English. "Pointing helps," she says, "I show by example." In places such as South Africa, where there are 11 languages and a severe teacher shortage, the labs rely heavily on students to show the next set how to use machines. "That turns out to be a really powerful tool for Fab Labs," she says.

And why not? There is little else for these students to do. In many African countries, there are not many jobs for people with these mid-level skills. At one of Rwanda's few technical schools, for example, secondary-level students — about a third of whom are orphans of the 1994 genocide — earn a three-year diploma in topics such as automotive mechanics, electronics or construction. But of the 105 students at the Gitarama school who graduated last year, says the principal, Mark O'Kane, only 20 have found jobs, and 8 of those are with the school.

School officials are now talking to Rwandan companies to try to train more employable students. Gershenfeld has something similar in mind. A virtual Fab Academy could equip students with skills that would make them more attractive to local companies, he says, particularly in countries with growing technology sectors such as India and South Africa. Local companies interested in employees with certain skills could also sponsor a lab. A handful of companies, including Honeywell India's training centre at Madurai in Tamil Nadu, and Neuron Bio of Pune, Maharashtra, are negotiating support for new labs there.

Raising funds to maintain the labs will be the project's biggest test. Infrastructure challenges occur at every level, says Sun, from renting a truck to move the machines and keeping the labs stocked with consumables, to ensuring electricity or Internet access, and providing technical support if a machine breaks down.

Lab staff are usually either volunteers — akin to a "technology peace corps", says Gershenfeld — or shared with other community



Rich variety: Haakon Karlsen holds a radio-tracked sheep, and Neil Gershenfeld presents pre-teen research.



R. FRIEDMAN/CORBIS

projects. But each lab still costs as much to run as a small business in the host country. So far they have been supported by a mixture of funds from MIT, local governments and grassroots groups. But as the number of labs grows, Gershenfeld has struggled to convince traditional development agencies to finance them.

He now has interest from several private donors, however, and is launching the Fab Foundation to match these donors to labs and coordinate the growing network so that existing labs can learn from each other and new ones can grow more easily. It's already possible, he points out enthusiastically, for a Fab Lab to make buildings and furniture, and so "we're at the point of physically making the lab in a Fab Lab," although new machines would still have to be shipped from MIT.

Into business

After much debate, the Fab Labs have together decided that anything made in one lab should be available to the others. In this way, the focus is less on intellectual property — which is hard to protect in many developing nations — and more on making the business model suit the people doing the work, the invention and the lab's location. In theory, an invention from one lab could be made by several others, essentially creating a global collection of local businesses. Gershenfeld hopes to help by providing small amounts of capital from a 'Fab Fund'. The main expectation, he says, is that there is a return for the community.

But ensuring that return is the tricky bit. Even when they have good ideas, "taking them to the next level takes a lot of business understanding", notes Behrokh Khoshnevis, director of the Center for Rapid Automated Fabrication

Technologies at the University of Southern California in Los Angeles. "Competing with industry is a big, big challenge." Khoshnevis says that for the Fab Labs, "the focus should be on education more than anything else".

Khoshnevis can appreciate their value better than most in the West: as a nine-year-old in Iran, he couldn't afford fancy toys and built himself a four-storey polystyrene building equipped with a working elevator the size of a cigarette box, complete with doors and lights. If there had been a Fab Lab to teach him, he says, his inventions could have matched his imagination.

So far the labs have excelled at recreating the outreach experiment that got them started. The Fab Village in Norway has been so successful at engaging the community that there are plans to start others in Iceland, Sweden, Finland and Russia. The lab in Ghana is similarly set to help launch labs in Kenya and Rwanda.

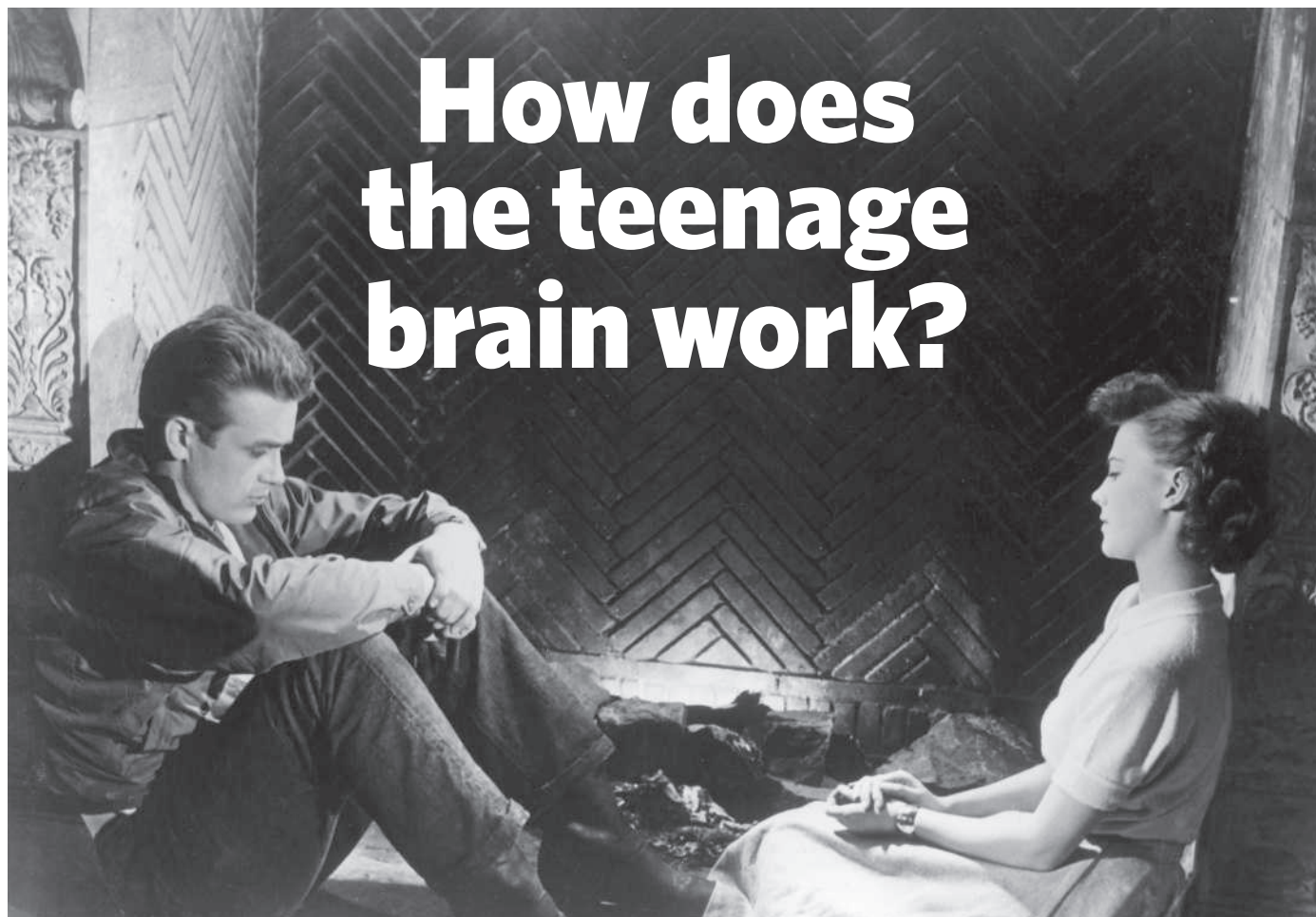
There is more to this story than philanthropy, though: the innovatory echoes of the outreach project have bounced back to MIT. Again, it was an eight-year-old girl who showed the way: Gershenfeld's daughter, Grace. Last year Gershenfeld was showing her how to use a laser cutter in the MIT lab, but Grace thought the shapes he was cutting out were boring. Instead she made a cardboard 'Lego kit' of two-dimensional pieces that snap together to form three-dimensional objects.

Playing with it, Gershenfeld realized the flat shapes let him build space-filling parts, "That hadn't occurred to us as a way to make stuff at MIT." He and his colleagues are now testing ways to scale this approach up or down, by using parts of various sizes and in different materials. "There are now up to four grad students developing the work of an eight-year-old kid," says Gershenfeld. As Karlsen says, "We are clever people wherever we live. You see that we can do anything."

Apoorva Mandavilli is senior news editor for *Nature Medicine*.



The defence of personal space inspired one US student's work.



KOBAL COLLECTION

How does the teenage brain work?

Changes in the structure of children's brains may account for some of the risky business of adolescence, **Kendall Powell** finds.

The 14-year-old has a very simple decision to make. When he sees a light out of the corner of his eye he is supposed to ignore it and keep looking straight ahead. It seems extraordinarily easy — even eight-year-olds can do it correctly half of the time — but it requires reigning in a natural impulse to look. And every parent of a teenager knows that reigning in impulses is not their strong suit.

In this simple test, the teenager performs as well as adults do. But a peek inside his head reveals that he puts a lot more work into it. His brain uses a whole host of frontal regions — those involved in planning and executing actions — that adults ignoring something in their peripheral vision just don't need.

"The adolescent brain is acting like an adult brain doing something much more difficult. An adolescent can look so much like an adult, but cognitively, they are not really there yet," says Bea Luna, a neuroscientist at the University of Pittsburgh Medical Center in Pennsylvania. It is her brain scans that have revealed this tendency for teenagers to 'overuse' their frontal brain regions when stopping themselves from looking at the light¹.

Work such as Luna's begins to explain why a teenager can behave maturely on Monday and

do something unutterably foolish on Tuesday. Neuroscientists probing the teen brain have found that it undergoes a major remodelling that may be responsible for the teenager's propensity to take risks, seek out new experiences and fail to restrain inappropriate responses. Their work suggests that, even before you add raging hormones and peer-group-driven rebelliousness-without-a-cause to the mixture, teenagers may simply be unable consistently to make decisions the same way adults do. This could well be one of the reasons that, although most people are healthier during their adolescence than at any other time in their lives, adolescents are three or four times more likely to die than children past infancy²: they take risks, have accidents and pay the prices.

The teenage years turn out to be a complicated time in the brain, with cells fighting it out for survival and the connections between different regions being rewired and upgraded. Some abilities, such as quashing offensive behaviour and empathizing with others, keep maturing well into the twenties. The passage from childhood to adulthood is not straight-

forward: some researchers now see the teenage remodelling as analogous to the 'developmental window' that allows the brain to be moulded by experience in infancy. There are ways in which teenage brains perform quite differently from either childish or adult ones.

No one is saying that a desire to yak endlessly on the phone, sneak out of the house or throw yourself off the side of a mountain on a

snowboard in the gnarliest way possible is purely a function of neural architecture. But the brain changes tracked by neuroscientists offer parents, teachers and any other caring adult insights in how to help teenagers avoid too much danger. And such studies look likely to enrich scientific explanations of

teenage vulnerability to depression, addiction, eating disorders and schizophrenia.

By around the age of 12, a child's brain has the size, folding, weight and regional specialization of an adult's. But a decades-long study conducted by the US National Institute of Mental Health (NIMH) in Bethesda, Maryland, has shown that such a brain still has a long way to go to reach adulthood. Begun in 1991, the study has followed 2,000 people who were then aged

"An adolescent can look so much like an adult, but cognitively, they are not really there yet."

— Bea Luna

from 3 to 25, taking brain scans every 2 years. The study uses magnetic resonance imaging (MRI) to measure the water-to-fat content of tissues: in the brain, this distinguishes grey matter, which is mostly water-filled nerve cell bodies, from white matter, which is mostly made up of the nerve connections sheathed in fatty insulation called myelin.

The NIMH research team, led by Jay Giedd, has made a movie of normal brain changes from ages 5 to 20 (ref. 3). It reveals that the grey matter thickens in childhood but then thins in a wave that begins at the back of the brain and reaches the front by early adulthood (see graphic, below). The process completes itself sooner in girls than in boys. This corresponds to a long-held assumption that adolescence sees the prefrontal cortex regions that handle executive functions 'waking up' and to the conventional wisdom that girls mature faster in this respect.

But, Giedd says, "the real message is that what is important is the journey, not the destination". His studies and others show that measuring the shape or size of a certain region in an adult can mislead: what really counts is the developmental trajectory that gets you there. Earlier this year, the team showed that a more pronounced wave of grey-matter thinning corresponds to higher intelligence⁴.

Use it or lose it

Giedd and many other neuroscientists think the grey-matter thinning seen during adolescence is probably due to 'synaptic pruning' — the process of eliminating overabundant, unnecessary nerve cell connections. If synaptic pruning is accelerated during adolescence, says Giedd, it follows that this is a time of 'use it or lose it' in the brain. The more environmental input there is to guide that pruning, he says, the better. On the same basis he argues that less guidance could result in a brain less able to react to complex situations, as could uncontrolled pruning: preliminary studies show that childhood schizophrenics have an exaggerated



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Functional MRI scanning requires this teenager to adopt a favourite position.

loss of grey matter during adolescence⁵.

Giedd takes this to imply that teens should be exposed to rich environments full of sports, travel, music and foreign languages. In the past decade, most efforts to provide brains with extra stimulation focused on the first five or six years of life. This new work, says Giedd, should argue for an equally critical period of brain plasticity in adolescence.

However, Elizabeth Sowell, a neuroscientist at the University of California, Los Angeles, cautions against making direct connections between brain changes and specific teen behaviours. "Jay likes to say 'use it or lose it' and that we should put kids in enriched environments. That makes perfect intuitive sense, but we just don't have the data to say that."

Tomáš Paus, director of the Brain and Body Centre at the University of Nottingham, UK, adds a further note of caution, warning that researchers should be careful not to simplify the brain-behaviour relationship into a one-

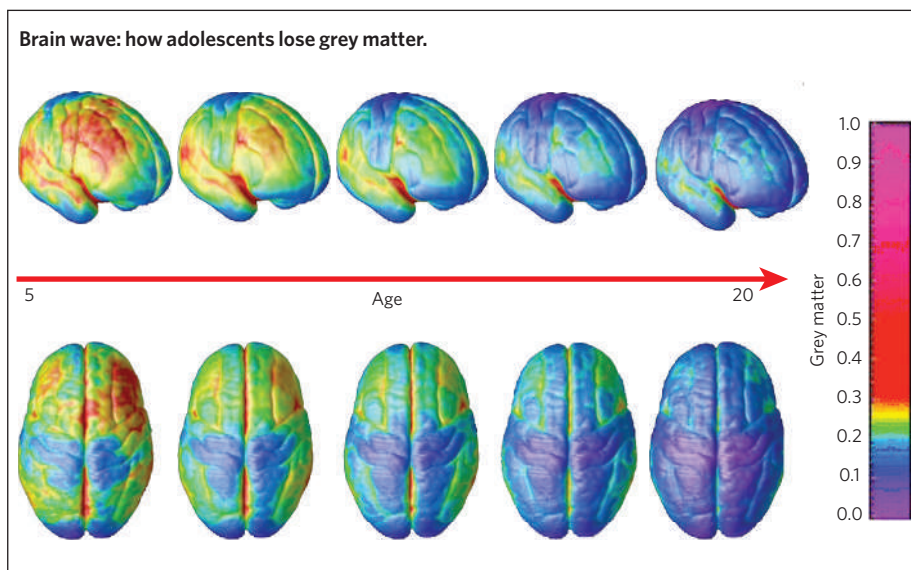
way street. Behaviour almost certainly influences final brain structure, too; indeed, this is what Giedd's current research is looking at. But Paus agrees the research has shown that the "brain continues to mature way beyond the first 3 to 5 years of life". Referring to a popular pre-school 'enrichment' programme in the United States, he says: "We sorely need a Head Start programme at the beginning of adolescence. Things are not carved in stone by that age. Our field's observations show that you can induce long-lasting changes that are entered into the brain."

As grey matter thins, white matter is being gained, as layers of insulating myelin are added to the axon connections between nerve cells. George Bartzokis, a neuroscientist at the University of California, Los Angeles, has found that such 'myelination' follows an inverted 'U' shape over our lifetimes, peaking at around age 50 (ref. 6). The teen years are on the early stages of the steep upward curve of myelination.

Bartzokis sees this as facilitating connections between different parts of the brain: if you want to retrieve pertinent information quickly to make a decision, he argues, you don't want a supercomputer "but rather a fast Internet". Information held in different centres of the brain has to be 'online' or retrievable, and retrieving lots of it quickly requires increased processing speed and bandwidth. Myelination provides this by increasing the speed of signals travelling along axons and decreasing the time to the next nerve impulse.

"Two years from now, my daughter will be 16, and my car insurance bill will double," notes Bartzokis, even though she probably knows the rules better and has quicker reflexes than he does. "But she will still have a much higher risk of getting into an accident because she can't instantly bring knowledge to bear in a situation like I do." What we call wisdom, Bartzokis says, requires maximum myelination.

J. GIEDD





G. PALMER/CORBIS

Listen to reason? He might not be able to.

Abigail Baird, director of the laboratory for adolescent science at Vassar University, in Poughkeepsie, New York, and her graduate student Craig Bennett mapped white matter in the brains of first-year undergraduates and then looked again six months later to see if anything had changed. They found subtle but significant additions: five brain regions gained white matter, including frontal areas that prepare for action and form strategies, and other areas that integrate sensory input, emotions, body state and context⁷. A control group of postdocs showed no such changes. "It's the stuff that allows you to put yourself in another's shoes or have empathy in the broad sense," explains Baird.

But she adds a strong note of caution about her work — it only shows a correlation between brain development and the ability to empathize. "We can't prove that the changes we saw do anything to cause certain behaviours. It's important to remember that this is a little bit of a 'Just So' story."

Brain at work

If the adolescent brain differences were only a matter of getting the correct grey-matter-to-white-matter balance, the story might end here. But the plot thickens with the work of Luna's group, which uses functional MRI (fMRI) to measure activity in different regions of the brain by tracking blood flow. Connecting her work on the 'overuse' of the teenager's frontal lobes to Giedd's ideas, Luna associates the onset of adulthood with an integration of the frontal regions with other areas of the brain — a move from local-area networks to wide-area networks, in computer terms. Myelination is probably part of the process, but not all of it: if it were, peak brain efficiency would not be seen until late middle age.

Other functional studies link changes in the brain to teenagers' increased appetite for fast

cars and other dangerous thrills. B. J. Casey's group at Weill Medical College of Cornell University in New York has measured brain activity in subjects who perform a simple task and then get small, medium or large rewards for performing correctly. In adolescents given a medium or large reward, a centre in the brain called the nucleus accumbens reacted more strongly than in children or adults⁸. That looks like an exaggeratedly positive reaction. When given the small reward, the teenage accumbens response decreased below that of children and adults — as if the small reward represented no reward at all in the teen's view.

Like Luna, Casey sees a more diffuse pattern of activity in the teenage frontal regions than in those of adults. A reward centre on overdrive coupled with planning regions not yet fully functional could make an adolescent an entirely different creature to an adult when it comes to seeking pleasure.

"If it were just that the prefrontal cortex was lagging behind, then children would be as risk-taking as teens, and we don't see that," says Casey. She goes on to speculate that the lag between the frontal regions and the reward centre is an evolutionary feature, not a bug. "You need to engage in high-risk behaviour to leave your village and find a mate," and risk-taking soars at just the same time as hormones drive adolescents to seek out sexual partners. Linda Spear, a behavioural neuroscientist at the State University of New York at Binghamton, says that in rodents, primates and even some birds, adolescence is a time of risky business, seeking out same-age peers and fighting with parents⁹, which "all help get the adolescent away from the home territory".

The growing evidence that increased risk-taking is wired into the adolescent brain is beginning to shift the way psychologists

approach 'troubled' kids. "I don't think we can fight the biology of wanting to take risks and try on different identities. Those are good things," says Giedd. "But how do you have outlets that don't give teens STDs, car accidents, drug abuse or jail? As a society, we can give kids creative, positive outlets that do not lead to irreversible mistakes." Attempts to push kids towards safe sex or pharmaceutical temperance shouldn't be expected to succeed if they simply explain consequences. "Adolescents have some fundamental qualities to them that are not voluntary and not easily modified by rational, information-based interventions," says Laurence Steinberg, a developmental psychologist at Temple University in Philadelphia.

Can't wait

And for some kids 'just saying no' will be even harder than for others. Casey, in collaboration with Inge-Marie Eigsti at the University of Connecticut in Storrs, has measured the ability of 4-year-olds to defer gratification by offering them extra cookies if they could leave a first one uneaten for a set period of time. When tested 10 years later, the kids who resisted temptation as pre-schoolers were better at restraining themselves as teenagers¹⁰. When children are predisposed to listen to their pleasure centres "and then the same system becomes elevated during adolescence", says Casey, "it's a double whammy. Those are the kids we are trying to identify."

Children who show early problems with focusing attention, Casey thinks, may need stronger parental guidance later. But in general she and her colleagues shy away from making specific recommendations on the legal responsibilities, parenting or education of teenagers.

Instead, they tend to emphasize the newfound appreciation for adolescent behaviour their work offers. The next time your quiet reverie in a café is interrupted by a gaggle of teenagers getting their after-school caffeine and sugar fix, as this writer's has just been, take a moment to remember the teenage boy in the MRI scanner, his earnest brain working overtime just to restrain a simple flick of the eyes. Hard work is going on inside those heads. ■

Kendall Powell is a freelance science writer based in Broomfield, Colorado.

**"It's important to remember that this is a little bit of a 'Just So' story."
— Abigail Baird**

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Gender: missing the prizes that can inspire a career

SIR — I congratulate Ben A. Barres on his excellent Commentary “Does gender matter?” (*Nature* **442**, 133–136; 2006). I was struck by the paucity of female plenary lecturers at the Bioscience 2006 meeting of the UK Biochemical Society. Spurred on by Barres’s comment that too few women academics speak out against prejudice, I decided to do a little research on the matter.

There have been three meetings of the Biochemical Society in the new annual meeting format (Biosciences 2004, 2005 and 2006) and at these 1 of 10, 0 of 10 and 0 of 7, respectively, of the plenary lectures were given by a woman. Some of these plenary lecturers were recipients of prizes and medals, and I was so shocked by these statistics that I made a rough count of the proportion of women who have received these prizes over the years, as published on the society’s website at www.biochemsoc.org.uk. Recipients’ initials, rather than first names, are given, so I may conceivably have misattributed the male gender to some of the earlier names.

The prizes include the annual Colworth medal, given to a promising scientist under 35: only one has been awarded to a woman, out of 44 recipients, between 1963 and 2007. The statistics for the other prizes, up to 2007, are the Novartis medal, 2 of 39; Jubilee lecture, 1 of 23; Wellcome Trust award for research in biochemistry related to medicine, 1 of 11; AstraZeneca prize, 1 of 5; Frederick Gowland Hopkins memorial lecture, 0 of 24; Keilin memorial lecture, 0 of 21; Morton lecture, 0 of 14; Biochemical Society medal, 0 of 3; and GlaxoSmithKline medal, 0 of 2. This translates into 3.2% of the prizes being given to women, a truly lamentable record.

Furthermore, the statistics have not improved. In the past ten years, none of the Colworth medals has been awarded to women — and it is prizes such as these, given to scientists early in their career, that influence their future success. The results speak for themselves: that people will always give prizes to others in their own image, unless forced to take sexual and racial bias into account. I wonder if the record of other scientific societies is much better in this regard.

I should also point out that UK Biochemical Society meetings are supported by funds from the Biotechnology and Biological Sciences Research Council and by the European Molecular Biology Organization. Why do research funding bodies not assert leverage on this matter, by insisting that sexual and racial bias in

speaker selection must be addressed at any meeting for which their financial support is given?

Annette C. Dolphin

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Gender: macho language and other deterrents

SIR — In the Commentary article “Does gender matter?” (*Nature* **442**, 133–136; 2006), Ben A. Barres cites our article pointing out that the first round of the US National Institutes of Health (NIH) Pioneer awards was carried out in a way that would have predicted a bias against selection of women (M. Carnes *et al.* *J. Womens Health* **14**, 684–691; 2005). Indeed, no women were selected in the first year, so when 43% of the second year’s winners were women we examined the process again to see what had changed.

We identified several differences, including changes made by the NIH, that would predict a decrease in the activation of automatic gender stereotypes that may have discouraged women from applying and disadvantaged women applicants in the first round.

First, a reduction in the number of applicants (from 1,300 to 840) and greater familiarity with an application process that was no longer new may have reduced time pressure on the reviewers.

Second, the NIH removed the repeated mention of the need for applicants to engage in ‘high-risk’ research; we believe that this terminology encouraged male and discouraged female applicants. Similarly, the emphasis on ‘intrinsic’ leadership abilities and ‘potential’ of the scientist was removed, in favour of an emphasis on the scientist’s research.

Third, there was a much higher proportion of women in the applicant pool, which may have been related to the change in language (26% in phase 1 and 35% in phase 2 in 2005, compared with 20% and 10% in 2004). There was also a greater proportion of women on the review panel: 44% in 2005, compared with 6% in 2004.

Fourth, the presence of accomplished women scientists on the review committee provided a positive role model for applicants.

Finally, women were specifically encouraged to apply — a particularly significant factor in the context of the outcry in the scientific community following the absence of women in the first round.

We applaud the NIH for taking an evidence-based approach. Regardless of the gender composition of the group selected in the forthcoming third round, removal of conditions that are known to activate

automatic gender stereotypes ensures that the best science will be supported, regardless of the sex of the scientist.

Molly Carnes

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See *Nature* **442**, 510 (2006) for other letters on this topic. Readers are encouraged to add their comments on the Nature News Blog at: http://blogs.nature.com/news/blog/2006/07/does_gender_matter.html

A positive definition of prokaryotes

SIR — In his Concepts essay, Norman R. Pace argues that the concept of prokaryotes is misleading and proposes that the word ‘prokaryote’ be banned from the scientific literature¹. We disagree.

Pace contends that the term prokaryote refers to the lack of a nucleus and that it is hence a “negative and therefore scientifically invalid description” of cell organization, because “no one can define what is a prokaryote”. The former is a matter of opinion, and the latter is arguably incorrect.

Prokaryotes are cells with co-transcriptional translation on their main chromosomes; they translate nascent messenger RNAs into protein. The presence of this character distinguishes them from cells that possess a nucleus and do not translate nascent transcripts on their main chromosomes². Although historically founded on a negative trait (lacking a nucleus), the term prokaryote does indeed specifically designate organisms that are defined by a positive character.

Pace proposes that we should speak only of archaea and bacteria instead of prokaryotes, and that if a collective term is needed to designate those cells that are not eukaryotes, the term ‘microbe’ should be used. That suggestion, too, is unacceptable, because many eukaryotes are microbes.

Regardless of what any gene tree might suggest and regardless of what anyone might believe about early evolution, modern cells lacking spliceosomal introns and spliceosomes², a nucleus, and mitochondria³ do possess transcriptionally coupled translation — they are prokaryotes⁴.

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BOOKS & ARTS

Solving an age-old problem

Western governments need to rethink their approach to dealing with an ageing population.

The Denial of Aging

by Muriel R. Gillick

Harvard University Press: 2006. 328 pp.

\$25.95, £16.95, €24

John Grimley Evans

US citizens spend \$6 billion a year on anti-ageing nostrums. This reflects a general failure in society to acknowledge the realities of old age, believes Muriel Gillick, associate professor of ambulatory care and prevention at Harvard Medical School. Americans, she asserts, squander resources on quackery and on futile but expensive treatments for people approaching the end of their lives, and risk not leaving enough money for more beneficial care.

In the first part of her book *The Denial of Aging*, Gillick systematically dissects various medical and social provisions for older people in the United States. By linking each critique to a telling case history, she shows how even the best intentions of bureaucrats and politicians fail through not being implemented as part of an informed and coherent sociomedical strategy. Some of the problems are familiar enough to Europeans, notably the impossibility of providing seamless care from budgets separated into 'social' and 'health' elements, with a range of providers all trying to cherry-pick the easy bits and play pass-the-parcel with the rest.

The central problem, however, is one of philosophy: what are we trying to do for older people, collectively and individually? Governments simply want them — or at least those who have not funded their own pensions — to go away. The recent flaccid response of the British government to a critique of UK research into ageing by the House of Lords Select Committee on Science and Technology could be interpreted as anxiety not to encourage anything that might lengthen the lives of the improvident.

British geriatric medicine has always concentrated on the quality of life, rather than its length, and older people in Britain are more worried by the prospect of 'being a burden' than of being dead. US medicine, by nature of its funding structure and the legal and social pressures under which it labours, is more than appropriately preoccupied with prolonging life at any cost. There is a gap in medical resources and philosophy between this frenzied use of high technology and the resigned fatalism of palliative care. Between the life-support machine and the morphine pump, there is a lot that can and



D. LECORRE/ALAMY

Forever young? Rather than face up to old age, many US citizens try to delay it for as long as they can.

should be done for ill older people, but appropriate prescription requires the setting of individualized goals and an individualized appraisal of quality of life. Wilful and determined older people can demand and obtain the care they need, but many are simply rolled through a system created by piecemeal legislation and the profit logic of privatized providers.

Quacks flourish, but Americans also look to real science to relieve them from the terror of senescence. In consequence, biological gerontology has been tainted by a Californian dream of indefinite longevity. Perhaps eternal life seems a better idea in California than it might in Brixton. But even if the Colorado River keeps flowing and the San Andreas fault holds together, it is an idea doomed by the second law of thermodynamics and is a dangerous distraction from more important issues. All developed countries face a new demography of increasing life expectancy and falling birth rates. What we should concentrate on is the prevention and prompt alleviation of disability so as to maximize the productivity of the ageing population and the self-sufficiency of individuals. This calls for a new collaboration between biological and medical gerontologists working towards common and explicit objectives. Postponing the onset of disabling disease and slowing the background loss of adaptability that makes

pathogenic events more challenging may not necessarily increase life expectancy, but should increase disability-free life expectancy.

Rather than adjusting the institutions of society to a new trajectory of life, politicians are tempted by the plausibly easier option of demographic engineering. Gillick suggests that increasing the permeability of the Mexican border might alleviate a looming shortage of care workers in the United States. This may seem like a quick fix, but it would be damaging to Mexico and have little effect on longer-term demographic prospects for the United States. Immigrants grow old too, and their children will not be content to be cheap labour. Immigrant labour is even less of a good idea for areas where population density has outgrown both its water supply and its social tolerance, such as southeast England. Ageing populations in the West should create their own destiny, not prey on the underdeveloped world.

Gillick's book raises important issues in a lucid and accessible style. The reader cannot help feeling that the problems of ageing and longevity could be effectively dealt with by informed and intelligent political leadership. But if we had that, no one would have invaded Iraq.

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A walk in a quantum world

Fantastic Realities: 49 Mind Journeys and a Trip to Stockholm
by Frank Wilczek

World Scientific: 2006. 522 pp. \$21.98, £16

Michael Berry

The contemporary picture of the physical world is framed by quantum field theory (QFT), which combines quantum mechanics with Einstein's special theory of relativity. Frank Wilczek's mission to provide a non-technical account of QFT, aimed at physicists who are not specialists in particle theory, lies at the heart of his book *Fantastic Realities*. For non-physicists, however, the book would be hard going.

Wilczek points out that QFT explains three mighty facts about the world. First, that all elementary particles of each type (electrons, say) are indistinguishable from one another. This leads to a refinement of the philosophical concept of 'identicalness'. Second, that particles can behave in two ways when interchanged: the waves describing them can remain the same (bosons) or they can change sign (fermions). QFT can also explain why bosons are particles whose spin is an integral number of Planck units, whereas for fermions the spin is a half-integer (although this 'spin-statistics theorem' could well have a more elementary foundation). The third fact is that for each

particle there is a corresponding antiparticle, with opposite value for charge and other quantum numbers.

In this picture, the primary ('fantastic') realities are the quantum fields, and the particles — their elementary excitations — are epiphenomena. Wilczek argues persuasively for this interpretation, different from the view expressed by Richard Feynman, that particles are fundamental, and that QFT is the machinery (including what Wilczek calls "the hocus-pocus of renormalisation") for calculating how they behave. Different particles correspond to different fields. At the heart of the modern theory of matter is the field theory of quantum chromodynamics (QCD), which describes the elementary particles — quarks, with gluons mediating their interactions — that make up atomic nuclei. This is a more elaborate cousin of quantum electrodynamics, which describes electrons interacting by means of photons.

In essence, QCD is relatively simple to describe, but getting physical predictions out of it involves some fearsome calculations and fairly difficult concepts. Wilczek's own contribution to the field was to explain why quarks are never observed in isolation: they appear free at short distances but get tightly bound when separated. This paradoxical behaviour is associated with the concept of 'asymptotic freedom', which won Wilczek his share of the

2004 Nobel prize. The prize ceremony, and the curious culture surrounding it, is described at the end of the book in a blog by Wilczek's wife, Betsy Devine.

Although the book is largely non-technical, Wilczek's writing is deeply intellectual. The essays are centred around QFT but range far more widely. They display a sensitive appreciation of subtle features of the culture of modern physical science. In particular, Wilczek moves easily between the different levels of theory that describe different phenomena. This is 'reductionism', a term Wilczek hates because it connotes a diminution of our understanding, rather than an augmentation — or, to use Newton's phrase, 'analysis and synthesis'.

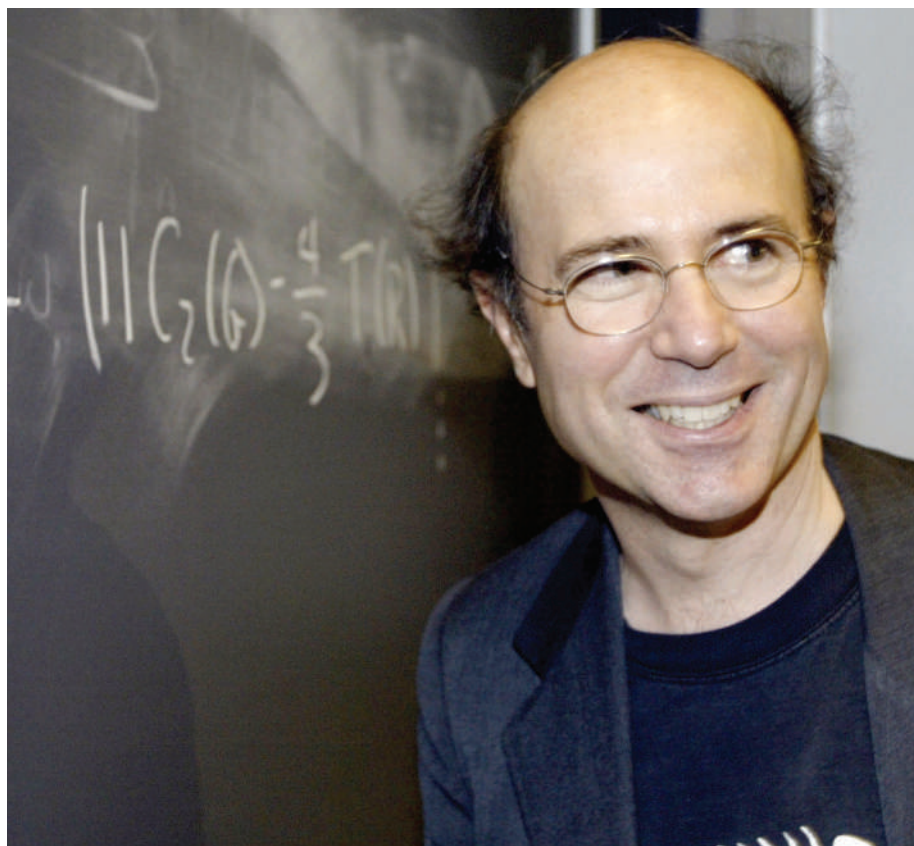
Wilczek describes a simpler version of QCD — "QCD lite" — in which the mass of quarks can be neglected. This explains many of the phenomena encompassed by the full QCD; in particular, it accounts for 90% of the mass of protons and neutrons, and therefore of ordinary matter. Thus most of our own mass corresponds to the energy of pure motion according to $m = E/c^2$, which as he points out was the original form of Einstein's iconic equation: "At this level, at least, we are ethereal creatures."

Another of the themes in Wilczek's book is the connections between high-energy theory and condensed-matter physics. These include phase transitions and collective quantum phenomena such as superconductivity and superfluidity ("the world is a multilayered, multicolored, cosmic superconductor"). In three-dimensional space, the only possibilities for identical particles are bosons and fermions. But for physics in two dimensions, there is, as one of the essays describes, a continuous range of intermediate possibilities ('anyons') with important applications in solid-state magnetism. I was disappointed by the absence of an account of Wilczek's astonishing work with Alfred Shapere, applying modern geometrical ideas to the swimming of small creatures and the reorientation of falling bodies such as cats.

Several essays cover essentially the same intellectual territory, but with such difficult concepts the inevitable repetition is no bad thing. In any case, it is fascinating to see Wilczek circling round the same set of ideas. There is much concentrated wisdom here, and he has a deft touch with words.

A delightful address to students contains Einstein's favourite joke and an algorithm for choosing between possible marriage partners. Here Wilczek betrays his incorrigible nature as a theorist, because the algorithm requires advance knowledge of one's likely lifetime number, N , of credible suitors. He does not advise the students how to behave when the algorithm fails (as it sometimes will), or when both partners apply the algorithm (probably with different N). But for Wilczek himself "it worked out fine". ■

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Attractive idea: Frank Wilczek won a Nobel prize for his work on the interactions between particles.

B. SNYDER/REUTERS

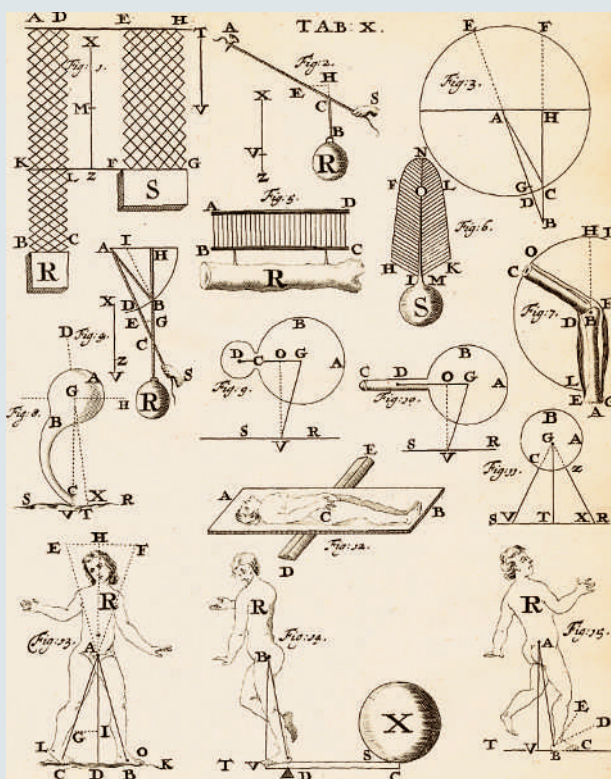
From mysticism to medicine

Science and Technology in Medicine by Andras Gedeon (Springer, €74.95) is an extensively illustrated selection of key works that introduced science and technology into medicine.

It begins at a time when medicine in Europe was mostly folklore and mysticism, with Albrecht Dürer's treatise in 1528 on human proportions, and closes with Michael Phelps' 1975 landmark paper on PET scanning.

A summary of each of 99 publications — the 100th is left to the imagination of the reader — is accompanied by a brief description of its author. The significance of the discovery and its influence on later medical developments are explained.

The illustration shown here is from the works of Giovanni Borelli and was published posthumously in his two-volume *De motu animalium* (*On the Movement*



of Animals) in 1680–81. Borelli adapted Galileo's science of mechanics to physiology, but realized that biomechanics

alone could not account for muscle contraction: chemical reactions within the muscle must be involved. **A.A.**

as cloves or bananas — presented a problem. Who to believe, other than one's own eyes? What 'knowledge' was reliable?

Because these men were trying to document what Douglas Adams called "Life, the Universe and Everything", nothing could be left out. To cope with this, the Renaissance *studiosi* developed a technique that we still use (and perhaps misuse) today: citation. Sources were carefully compared, dependent sources were acknowledged, and all were part of the description itself. This way, everyone knew where the information had come from, and could weigh and assess it themselves. But it was not enough just to trust authority blindly. Independent corroboration — a cornerstone of the modern scientific method — was also the central tenet of Renaissance natural history.

We already know that the methods we use today were invented by our antecedents, so what does this book have to tell today's scientists, working in the molecular and electronic age? Maybe not a lot in terms of the detail, unless you are fascinated by what other people did, as I am. But if you allow your mind to freewheel while reading the book, many resonances will begin to emerge. Issues we are coping with today were also issues in the Renaissance, such as standardization, coping with a superfluity of data and the limits of technology. Standardization allows collaboration: the Renaissance natural historians had a 'Republic of Letters'; we have multidisciplinary, multinational teams. Technology in the Renaissance was in part limited by human memory; today we have machines and computers that can do basically anything.

This book is not only about the development of a discipline in an exciting time, but about how science is done. The trajectory of science is never-ending, but what fascinates me is that so much that our predecessors did has come around again, albeit in a different guise. Yet the solutions to these problems are as varied as the communities in which we work. We are all limited in our own ways. Ogilvie uses the example of the sundew to illustrate how Renaissance botanists were blind to certain things — they never seem to have noticed the plant's ability to trap insects. We can laugh at this oversight now, but what are we missing about the behaviour of subatomic particles or transposable elements?

As a natural historian, I enjoyed Ogilvie's history of my discipline. After reading the book, however, I feel he has done more than just write about the Renaissance science of describing; he has written the story of how science constantly reinvents itself, seen through the lens of the pre-Linnaeans. I recommend this book to everyone: not only will you laugh at the descriptions of the walrus and the banana, but it will make you think in a different way about how and why you do what you do. ■

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A blast from the past

The Science of Describing: Natural History in Renaissance Europe

by Brian W. Ogilvie

University of Chicago Press: 2006. 385 pp. \$45, £28.95

Sandra Knapp

In his beautifully crafted book *The Science of Describing*, Brian Ogilvie shows that history has much to teach us. His detailed examination of how the science of natural history developed in the two centuries before Linnaeus has lessons for all scientists, not just biologists.

Natural history is often thought to be an old-fashioned, out-of-date discipline, but go to any scientific meeting on genomics and you will hear talk after talk about what might be called the natural history of the genome. We are in a new era of discovery extraordinarily similar to that of the Renaissance natural historians. Ogilvie's book throws up parallels by exploring the development of natural history, focusing on botany in the sixteenth and seventeenth centuries, when plants were better studied than animals and information about them was a currency in the scholarly world.

Humanist tendencies to value empirical experience over theory were critical to the development of what Ogilvie calls the "science of describing" — the accurate description and documentation of what Renaissance scholars observed. Ogilvie argues against the idea that there was a seamless line leading from medieval botanical herbalism to today's natural history, as many of us were taught. Instead, he makes the case that the Renaissance *studiosi* (mostly botanists) rejected the teaching of the ancients and the necessity of utility as a primary concern in their work. They were more concerned with documenting what they saw, and, importantly, with assessing the evidence, figuring out whether the descriptions they were given were accurate or not.

As European exploration of the world gathered pace, the number of new plants and animals to be described exploded. This expansion of experience coupled with the weighting of objects over texts meant that these men (and they were all men) were dealing with a fact-rich universe — just as we are today. Travellers' tales — second-hand descriptions of animals such as walrus and reindeer, or plants such

NEWS & VIEWS

EARTH SCIENCES

Signature required

L. Paul Knauth

Most geologists agree that Earth's atmosphere was oxygen-free until 2.4 billion years ago. But the latest sulphur-isotope measurements from sedimentary rocks suggest otherwise.

L. PAUL KNAUTH

Newly embraced scientific paradigms usually have their share of lingering critics, quiet sceptics and bewildered agnostics. Nevertheless, few doubt the recently established consensus that Earth's atmosphere was completely oxygen-free before a great oxygenation event 2.4 billion years ago. But on page 908 of this issue¹, Ohmoto *et al.* report data on sulphur isotopes in sedimentary rocks that seem to contradict this theory.

The history of atmospheric oxygen is crucial for understanding the origin and history of life, the evolution of the atmosphere and the peculiar nature of early sediments. Geological evidence has always leaned towards an oxygen-free — anoxic — early atmosphere, but was never conclusive. Strong support for this theory finally arrived when a special kind of sulphur compound was found in sedimentary rocks. This compound has a particular ratio of isotopes that indicates ultraviolet exposure, and has only been found in rocks more than 2.4 billion years old². This isotopic signature must have been created in an ancient environment that lacked an ozone layer. The absence of ozone would have allowed ultraviolet radiation to penetrate deep into the atmosphere, where it could interact with sulphur dioxide emitted by volcanoes, so forming new sulphur-containing products with distinctive isotopic ratios.

But this isotopic signature could only have survived and entered rocks if the reaction products had avoided recombination with oxygen. Such recombination occurs with even the tiniest amount of atmospheric oxygen, so the presence of this signature in old rocks is compelling testimony for an anoxic atmosphere before 2.4 billion years ago³. The isotopic data are robust, numerous and agree with the most common interpretations of the geological evidence, and apparently have no sensible alternative interpretations.

So far so good, but now imagine a lake 2.8 billion years ago, sitting beneath an anoxic, ultraviolet-drenched atmosphere. Sulphur dioxide emitted by distant volcanoes gets shattered in the atmosphere by ultraviolet light to form elemental sulphur and sulphuric acid, giving them a distinctive isotopic signature. These products mix into the lake water, where the sulphur settles along with small clay particles in

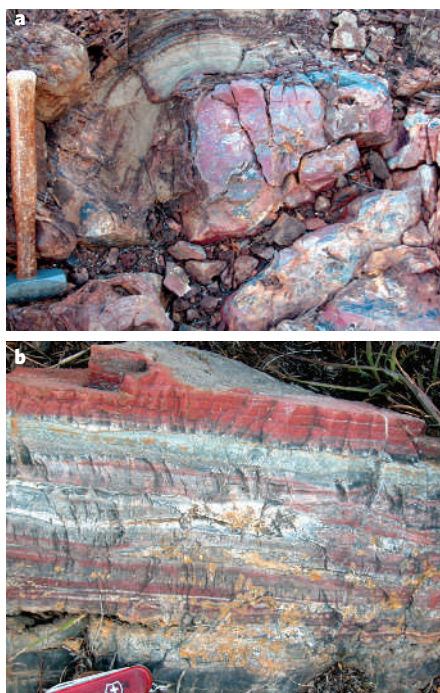


Figure 1 | Between a rock and a hard place. These oxidized sedimentary rocks formed at a time when the atmosphere is thought to have been oxygen-free. They may have arisen in special ways, such as at localized 'oxygen oases' where photosynthesizing organisms enriched waters with oxygen. But they could indicate problems, also raised by Ohmoto and colleagues' data¹, with the consensus that Earth's atmosphere was oxygen-free until 2.4 billion years ago. **a**, Strelley Pool chert at Pilbara Craton, Western Australia, is 3.43 billion years old, and is draped over older cherts holding oxidized iron. **b**, Sedimentary jasper beds in the Barberton Mountains, South Africa, are 3.227 billion years old.

quiet areas away from shore. As this sediment accumulates, iron reacts with the sulphur to make tiny crystals of pyrite (FeS_2), in a process well known to geochemists. Nowadays, streams that trickle into lakes contain sulphur from the Earth's crust in the form of sulphates, which are derived from the weathering of sulphur-containing minerals with oxygen. But this cannot happen in the anoxic atmosphere of our imaginary ancient world, so the pyrite begins its long

burial mostly free of the contamination that could obscure its atmospheric isotope signature. Aeons later, Ohmoto and colleagues drill into the deposit and measure the concentrations of the different sulphur isotopes¹. If our daydream is accurate, the ultraviolet-induced isotopic signature should be seen. But it isn't.

So what now? Perhaps more data should be obtained from the commonly analysed ancient seabeds. Unfortunately, ocean sediments may not display an atmospheric sulphur-isotope signature if they are close to seafloor hydrothermal vents. These vents emit large amounts of sulphur that carries a crustal distribution of isotopes, swamping any contribution from the atmosphere. Lakes, however, should be free of this hydrothermal activity, and so their sediments should contain sulphur derived mainly from the atmosphere, providing a good test for early atmospheric oxygen content.

Although the expected atmospheric isotope signature is absent from Ohmoto and colleagues' data¹, these do not give quite the normal isotope signature for crustal sulphur either. Perhaps the atmospheric signature is there, but much smaller than was anticipated. If so, the oxygenation of Earth's atmosphere must have occurred less than 2.4 billion years ago, because similar values for the isotopic signature show up in younger sediments and are not considered to indicate 'sunburnt' sulphur. Also, the authors have yet to measure levels of the heaviest sulphur isotope, which provides a double-check on the signature if isotopic variations are small. Such analytical fussiness was used originally to define the global atmospheric-oxygenation paradigm, so true believers still have room to question the authors' negative results.

Could the pyrite crystals sampled by Ohmoto *et al.*¹ have washed into the lake from somewhere else? Possibly, but they have the form of crystals that grew in place. Maybe hydrothermal fluids bathed the sediments with solutions rich in crustal sulphur, so forming the pyrite? Perhaps, but tell-tale cross-cutting veins and hydrothermal minerals are absent, undermining this possibility. The atmosphere present when the lake sediments formed remains the most likely source of sulphur for the pyrite.

Earth scientists may welcome one of the

authors' possible explanations of their findings — a 'yo-yo' atmosphere, in which oxygen levels rose and fell before the great oxygenation event. Beautiful Archean jaspers, rose-coloured cherts (Fig. 1) and other sediments older than 2.4 billion years that contain oxidized iron require special explanation if they formed in anoxic waters. Ultraviolet oxidation of surface waters offers one possibility, but this doesn't fit the geochemical evidence. Yet another theory involves isolated 'oxygen oases', where early photosynthesizers briefly enriched isolated pools with oxygen. But this conflicts with the widespread distribution of oxidized deposits. Geologists have long asked why periods of oxygenation could not have occurred in fits and starts, instead of in a great, single event. Atmospheric modelers are reticent on this⁴, but it would certainly help to explain the geology.

Finally, could the great oxygenation paradigm be wrong because there are other, unknown ways to make the ultraviolet-induced sulphur-isotope signature? For example, heating a mixture of sulphate and amino acids apparently produces a weaker version of the 'ultraviolet' signature, without using ultraviolet light⁵. Suggestions for alternative mechanisms usually induce howls of disbelief, but a similar

prejudice was challenged after the discovery of oxygen-isotope anomalies in meteorites⁶; the oxygen-isotopic signature was subsequently created experimentally in two completely unexpected ways^{7,8}. However, there is no known mechanism that excludes ultraviolet and that can still produce the large sulphur-isotope signature observed in old rocks.

Like much of the geological evidence, Ohmoto and colleagues' measurements¹ are not easily reconciled with the global oxygenation paradigm. Bewildered agnostics can rejoice. These data cannot be ignored, and the field has just become even more interesting. ■

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CELL BIOLOGY

A licence for duplication

Erich A. Nigg

Genome stability in animal cells requires strict control over the numbers of the organelles called centrosomes. An attractive 'licensing' model now explains how centrosome duplication is restricted to just once per cell cycle.

The centrosome is one of the most intriguing organelles found in animal cells. It organizes the construction and maintenance of the microtubule cytoskeleton, a molecular 'scaffold' that confers shape and polarity on the cell¹. Most strikingly, the centrosome duplicates during the cell-division cycle², and the duplicated centrosomes then help to form the spindle apparatus that segregates the duplicated chromosomes into the daughter cells. Because aberrant centrosome numbers can cause chromosome mis-segregation, such abnormalities have been proposed to contribute to the development of cancer^{3,4} — cancer cells often have abnormal chromosome numbers. The way in which centrosome numbers are controlled has been shrouded in mystery, but in this issue Tsou and Stearns (page 947)⁵ provide evidence supporting a simple control mechanism.

Every centrosome comprises two centrioles — barrel-shaped structures made of nine microtubule triplets — embedded in a complex protein matrix¹. So, from a mechanistic perspective, the problem of centrosome duplication essentially comes down to the question of how centrioles are duplicated. At the morphological level, the

centrosome/centriole duplication cycle is well understood². One notable aspect of the centrosome cycle is that parent and progeny centrioles show a tight right-angled (orthogonal) association (Fig. 1). This 'engagement'⁵ is established during duplication and persists through the subsequent phases of cell-cycle progression until 'disengagement' late in M phase and/or early G1 phase of the cycle separates the progeny centriole from its parent. So, during G1 phase, the two (future parent) centrioles are clearly separated, albeit loosely tethered to one another, and centriole disengagement has long been thought to be a prerequisite for duplication. However, the full significance of this peculiar process in the cell cycle is just beginning to emerge.

Three years ago, Wong and Stearns⁶ provided evidence that there is a block intrinsic to centrosomes that prevents the centrosome from duplicating again during late S and G2 phases — thus, it can duplicate only once per cell-division cycle. This implies that passage through M and/or G1 phases might be required to create permissive conditions — or 'issue a licence' — for a new round of centriole duplication in the next S phase.

Tsou and Stearns⁵ now use an *in vitro* assay based on frog (*Xenopus*) egg extracts and purified centrosomes to monitor simultaneously centriole disengagement and the growth of new centrioles under different experimental conditions. Their results strongly suggest that centriole disengagement requires the activity of separase, a protein-digesting enzyme previously shown to have a leading role in triggering the separation of duplicated chromosomes⁷. Furthermore, their data show that centriole disengagement is a prerequisite for the subsequent growth of new centrioles. This work provides an appealing model for how centriole duplication is normally limited to occurring only once in every cell cycle. In essence, centriole engagement (established during centriole duplication in S phase) prevents further centriole duplication until the activation of separase during M phase triggers centriole disengagement. Disengagement licenses the centrioles to undergo a new round of duplication (Fig. 1). This mechanism for limiting centrosome duplication to once per cycle is reminiscent of the controls that prevent multiple rounds of DNA replication.

Even landmark studies do not answer all the questions — on the contrary, they generally raise interesting new ones — and the work by Tsou and Stearns⁵ is no exception. Although the data strongly implicate separase in centriole disengagement, the evidence is mostly indirect. Definitive proof will have to await a direct and positive demonstration of the action of this enzyme. If separase does induce centriole disengagement, then how does it act? The simplest mechanism would be that it cuts one or more of the centriole-associated proteins that glue centrioles together (Fig. 1). Alternatively, it could act indirectly, for example by regulating the activity of another enzyme (a kinase or phosphatase, perhaps) that in turn triggers centriole disengagement.

These findings may have implications for the mechanisms that underlie the increased centrosome numbers typical of human cancer cells⁴. Deregulation or premature activation of separase might cause multi-polar spindle formation or create conditions allowing multiple rounds of centriole duplication in a single cell cycle. Alternatively, centriole numbers might be increased through loss of other controls yet to be discovered. Although the licensing model neatly explains how centriole duplication is limited to once per cell cycle, it does not explain how cells ensure that only one centriole is built next to each parental centriole. In the case of DNA replication, it is clear how each strand of the double helix forms a template for the synthesis of exactly one complementary strand, but how the growth of progeny centrioles is controlled is unknown. What prevents the simultaneous growth of two or more progeny in each round of duplication, for instance? And how can a cylinder form a template to grow a new centriole at a right angle to itself?

Centriole duplication is known to require

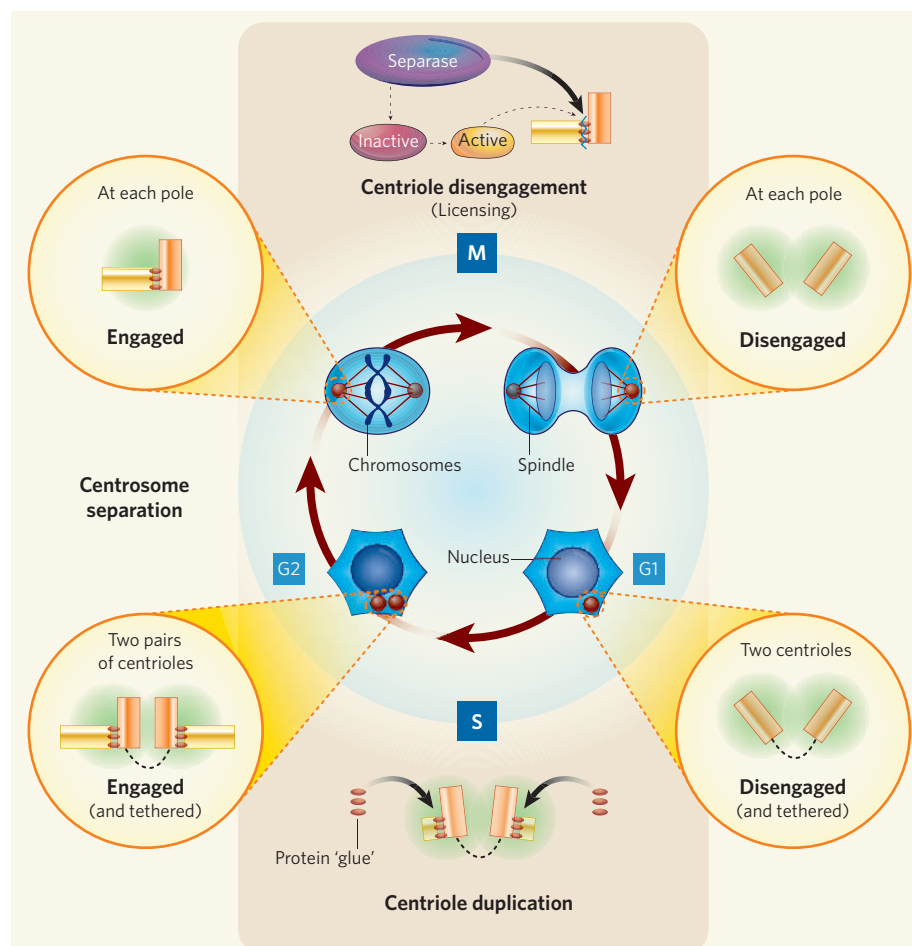


Figure 1 | Proposed role of the enzyme separase in triggering centriole disengagement. The central panel shows the centrosome duplication-segregation cycle. Each centrosome is shown as a red circle; G1, S, G2 and M are stages of the cell-division cycle. The arrangements of the centrioles at different stages of the cell cycle are shown in the insets. Centrioles are the orange and yellow cylinders; the pericentriolar protein matrix that surrounds them is green; a putative tether connecting parental centrioles is shown as a black dotted line; and the protein structure ('glue') presumed to be responsible for centriole engagement is red. Only disengaged centrioles are competent for duplication, whereas engagement prevents a second duplication. This 'licensing' mechanism thus ensures that centrioles are duplicated only once in every cell cycle⁵. Tsou and Stearns⁵ show that the enzyme separase is essential for disengagement of the centrioles. But it is not known whether separase acts directly on the glue proteins itself, or whether it activates some other factor that then divides the centrioles.

the action of several protein kinases that are active during S phase^{2,8,9}. From the perspective of centriole number control, it is intriguing that overexpression of the enzyme 'Polo-like kinase 4' triggers the simultaneous formation of multiple centriole precursors around each parent centriole⁸. So the study of this kinase holds promise for a better understanding of the putative templating function of centrioles.

Just as scholars of the centrosome cycle are beginning to reap these first rewards for their efforts, centrosomes are attracting the attention of an even wider community. Centriole dysfunctions have been implicated in a variety of human diseases, reflecting the role of centrioles (in another embodiment as so-called basal bodies) in the formation of cilia and flagella. These tiny, filamentous structures occur on the surface of many cell types and perform vital functions in normal physiology and development, including cell locomotion, movement of fluids or cells over epithelial surfaces,

sensory perception, and morphogenesis¹⁰. The centrosome/centriole is thus exciting the interest of researchers in a wide range of fields, and I anticipate many more discoveries about this enigmatic organelle.

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50 YEARS AGO

Tragic Safari by Albert Mahuzier—

This book is an account of a trip to French Equatorial Africa to make colour films of wild animals, especially at night by flashlight. All the plans went wrong, and the white hunter was mauled by a lion and bled to death for want of proper 'first aid'. The author has unusual ideas about filming animals in the field: he apparently aims at getting his pictures after the white hunter has mortally wounded the victims, or after he has caught a leopard in a 'panther trap', an enormous gin anchored to a log of wood... The value of the book may be judged by the caption beneath a photograph of the body of a warthog; it informs the reader that the picture shows a "dead rhinoceros".

The blurb on the jacket says "It is Mahuzier himself who gives us 'the loveliest girl of Maripanda', her beater raised in one hand, while the fingers of the other caress the taut tom-tom skin to produce the intoxicating double-rhythm". Reference to the picture, however, shows "the belle of Maribanda" pounding mealies in a wooden mortar with her free hand on the edge to prevent the contents spilling as she reduces them to meal. Further comment is needless.

From *Nature* 25 August 1956

100 YEARS AGO

A striking proof of the value of the finger-print method of identifying criminals is to be found in the recently issued report of the Commissioner of the City Police. During the past year 1,028 persons were arrested for offences under the Prevention of Crimes Act, such as being found in enclosed premises or in other circumstances suggestive of felonious intent. Of these individuals 562 were not recognised at the time of the arrest, but on their finger-prints being taken and compared with the Scotland Yard registers it was ascertained that 265 of them were old offenders.

From *Nature* 23 August 1906

50 & 100 YEARS AGO

NUCLEAR PHYSICS

Island ahoy!

Mark A. Stoyer

Measurements of the energy levels of unstable superheavy nuclei are beginning to afford distant vistas of a promised land — ‘the island of stability’ beyond the bounds of the conventional periodic table.

For nearly 40 years, nuclear theorists have predicted the existence of an ‘island of stability’ in the upper reaches of the chart of nuclides (Fig. 1). The island, a small region of longer-lived, superheavy nuclei jutting out from a sea of short-lived nuclei, is populated with nuclei that contain about 114 protons and 184 neutrons, and that reputedly live for anything from milliseconds to days. Over the past few decades, many different expeditions to the island — experiments designed to produce and observe its superheavy inhabitants directly — have been launched^{1–3}. But despite considerable effort, we are still eight to ten neutrons short of a direct landing.

Imagine, then, being able to infer the existence of the island of stability without ever actually going there; imagine having an eyeglass capable of peering towards the horizon of the nuclear landscape from the more familiar territory of lighter nuclei. On page 896 of this issue⁴, Herzberg *et al.* supply the first example of such an instrument through detailed spectroscopic measurements of the nucleus nobelium-254 (²⁵⁴No), which has a mere 102 protons and 152

neutrons. Their observations allow them to pinpoint, albeit tentatively, the location of the island of stability.

To understand how this approach works, one must first recall that, throughout the chart of nuclides, certain combinations of protons and neutron numbers seem to bestow enhanced stability on a nucleus. These ‘magic numbers’ are well explained by a nuclear-shell model incorporating the interplay of nucleons’ spin and angular momentum (so-called spin–orbit splitting), in which the protons and neutrons enter distinct shells as ever heavier nuclei are constructed. A nucleus is particularly stable when a shell is exactly full. This nuclear-shell model is very much analogous to the model of closed atomic electron shells in chemistry that explains, for example, why the noble gases are so disinclined to react.

The nuclear-shell model predicts that the next magic number of protons beyond lead (proton number $Z = 82$) would be 114. But various other extended and refined theoretical approaches disagree on the location of this shell gap: some support $Z = 114$, whereas

others predict $Z = 120$, 124 or 126. Some even predict no gap at all, instead suggesting a region of evenly spaced levels where enhanced stability might be washed out⁵.

It’s worth keeping in mind here that most of these models do predict a robust neutron shell gap at 184 neutrons. The stability (or lack thereof) of a superheavy nucleus comes from the interplay between proton and neutron shell structures, and differing nuclear shapes may also promote enhanced nuclear stability in this region⁶. Although all theoretical approaches reproduce known nuclear levels in the lighter mass regions more or less equally well, they are required to extrapolate very far to reach the island of stability. Identifying any nuclear energy levels in the region around proton number 114 would therefore further constrain the models, improving their predictive capability.

Herzberg and colleagues’ spectroscopic measurements⁴ of the decay of excited states in ²⁵⁴No, which they produce by reacting calcium-48 and lead-208 nuclei (Fig. 1), allow them to study the nucleus’s underlying structure, and in particular identify the specific single-particle levels responsible for several excited and metastable states. The excited states decayed to the ²⁵⁴No ground state by emitting γ -rays, X-rays and both primary ‘conversion’ electrons and secondary electrons, known as Auger electrons, that can be emitted when an electron from a higher level falls into the vacancy left by a conversion electron. Once the ground state had been reached, this too decayed through the emission of an α -particle.

This characteristic α -emission established the decaying nucleus as ²⁵⁴No, and the time distribution of emitted electrons is a signature of the decay of a metastable excited state that can be used to measure its lifetime. Herzberg *et al.* were able to reconstruct the energy levels of the nucleus from the energies of the conversion electrons and γ -rays, and could determine the spins and parities (whether the sign of the state’s defining wave functions changes when the sign of all spatial coordinates are flipped) of many of these states by characterizing the electric and magnetic nature of the γ -rays.

In addition, by measuring the g -factor (a measure of the magnetic moment) of one of the states, the authors were able to identify it as a two-proton excitation involving proton states that lie above the shell gap at proton number 114. This nicely sets the location of a single-proton level that will influence the superheavy-element region and the island of stability.

Additional metastable levels around this energy range are expected in neighbouring nuclei, such as the slightly lighter ²⁵²No, that are more difficult to produce than is ²⁵⁴No. Finding such levels would confirm this initial measurement and strengthen the systematic understanding of the nuclear levels responsible for the island of stability. Such observations from afar should in turn aid predictions

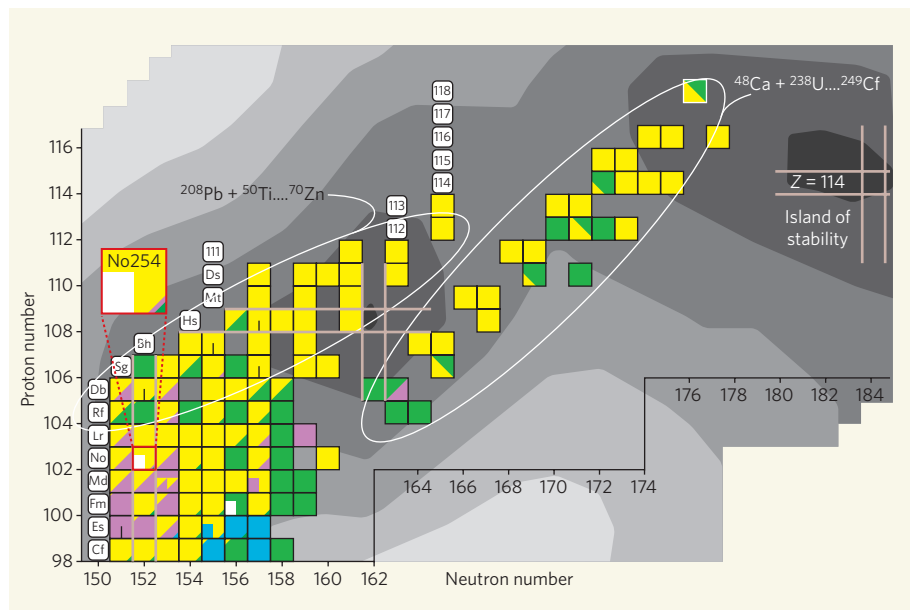


Figure 1 | Here be stability. The as-yet undiscovered ‘island of stability’ is proposed to lie in the upper reaches of the nuclide chart, around proton number (Z) 114 and neutron number 184. This is an otherwise complex nuclear landscape: coloured squares indicate nuclides that have been synthesized in the laboratory through nuclear reactions such as those between targets of lead-208 or uranium-238 and beams of lighter nuclei (left and central ellipses, respectively). Currently known instabilities to α -decay (yellow), electron capture (mauve), β -decay (blue), γ -emission (white) and spontaneous fission (green) are indicated, and contours indicate nuclide stability as predicted by the nuclear-shell model. Herzberg *et al.*⁴ identify nuclear energy states around the island of stability by synthesizing higher-energy metastable states of the nobelium-254 nucleus. (Figure courtesy of JINR, Dubna, Russia.)

of the lifetimes of nuclei populating the region around the island. This will help to pinpoint the island's exact location, and so guide the landing of future experimental expeditions. These results could even influence the types of experiment to be performed, as nuclei predicted to endure for between hours and days will require novel chemical techniques to be observed, and those predicted to live for seconds to hours will require sensitive mass analysers to be spotted.

With results such as those of Herzberg and colleagues⁴, we have an additional bearing

towards the island of stability — but a long voyage remains.

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as proton–chloride exchangers rather than passive chloride channels. These results were mind-boggling, because 'secondary active' ion-exchange transporters (antiporters) were considered to be very different from 'passive' ion channels.

Moreover, chloride channels were previously considered to provide an excellent mechanism for increasing the required acidification of certain vesicles (lysosomes or secretory vesicles) by proton pumps known as V-type ATPases. These ATPases pump protons into organelles, acidifying them, and chloride channels would provide an electrical 'shunt' to prevent the build-up of a large transmembrane voltage. So there seemed no good reason why endosomal transporters should require proton–chloride exchangers rather than chloride channels.

Enter plant CLC proteins. Previous research⁷ showed that a mutation in the AtCLCa gene results in reduced nitrate accumulation in *Arabidopsis* roots and leaves. De Angeli *et al.*⁴ now show that the AtCLCa protein is targeted to the vacuolar membrane (also known as the tonoplast) that surrounds the large vacuoles in plant cells. In plants, vacuoles can occupy up to 90% of the volume of cells. And, like endosomes and secretory vesicles, plant vacuoles have proton pumps that acidify the vacuolar lumen (Fig. 1, overleaf).

Plant vacuoles are large enough for the intact vacuolar organelles to be directly subjected to patch-clamp electrophysiology. This means that ion-transport proteins can be investigated in an intact organelle membrane that should include most auxiliary and regulatory subunits. Through elegant biophysical analyses, De Angeli *et al.*⁴ reveal that, in normal (wild-type) plants, there is a large proton–nitrate exchange current across the vacuolar membrane (Fig. 1). They also show that two lines of *Atclca* gene-disruption mutants lack this current, providing evidence that the AtCLCa protein encodes a vacuolar proton–nitrate exchanger. Their analyses further suggest that the AtCLCa-dependent

PHYSIOLOGY

Nitrate at the ion exchange

Julian I. Schroeder

The distinction between CLC ion channels and ion exchangers has become blurred. The physiological role of CLC exchangers has been a mystery, but one function is evidently to concentrate nitrate in plant vacuoles.

The cell membranes of all self-respecting cells contain ion channels that allow the selective flow of, for example, sodium or potassium, or anions such as chloride, into and out of the cell. Only a couple of years ago, a startling discovery was made that certain predicted chloride channels — specifically, members of the CLC family — function not as passive chloride-selective ion channels, but as proton–chloride exchangers that transport protons and chloride anions in opposite directions across cell membranes^{1–3}. But a mystery has remained: why do these CLCs carry out the proton–anion exchange shuffle?

In this issue, De Angeli *et al.* (page 939)⁴ show that a proton–anion-exchanger CLC has a central function in the flowering plant *Arabidopsis thaliana*^{*}. They find that the protein concerned,

AtCLCa, acts as an exchanger for protons and nitrate anions (NO_3^-) in the membrane of plant storage organelles called vacuoles. Nitrate is an essential plant nutrient, and AtCLCa uses the prevailing proton gradient across the vacuolar membrane to store nitrate at 50 times the level typically found in the cytoplasm — a feat that would energetically not be possible using a simple nitrate channel.

Study of chloride channels was something of a backwater until 1990, when the founding member of the CLC family was cloned from the electric fish *Torpedo marmorata*⁵. Interest further increased with studies showing linkage of diverse genetic diseases in humans and mice to mutations in different CLC genes⁶. Surprisingly, first the bacterial CLC-ec1 protein¹, and then the human CLC4 and CLC5 proteins^{2,3} that are located in the membranes of vesicles known as endosomes, were shown to function

^{*}This article and the paper concerned⁴ were published online on 26 July 2006.

BIOCHEMISTRY

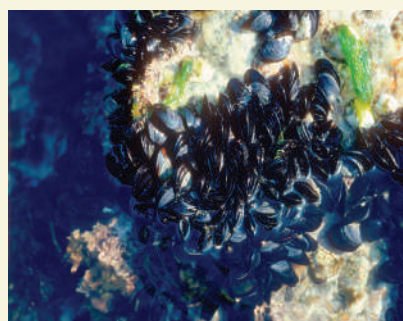
Mussel muscle

Anyone who has gathered mussels from the seashore will have been impressed by their ability to stick to rocks — even when lashed by waves.

Mussels can fix themselves to almost any organic or inorganic surface using a series of threads. Phillip Messersmith and colleagues (*Proc. Natl Acad. Sci. USA* doi: 10.1073/pnas.0605552103; 2006) now explain the role played in this gripping drama by an unusual amino acid named L-DOPA, which is found in at least five adhesive proteins in the mussel's threads.

Essentially, L-DOPA acts as the glue that sticks the adhesive proteins to surfaces. Using the technique of atomic force microscopy, the authors showed that L-DOPA molecules bind surprisingly strongly to a titanium surface, which is a good model for inorganic surfaces such as rock.

The combined effect of large numbers of these interactions is so great that if a whole mussel protein were pulled away from a surface, the protein itself would break apart



before the binding to the surface gave way. These interactions are reversible in ambient conditions, which implies that they cannot be covalent bonds.

In the marine environment, some of the L-DOPA units in the adhesive

proteins would become oxidized. Messersmith *et al.* show that oxidized L-DOPA binds weakly to titanium but apparently forms strong covalent bonds to organic surfaces. The solidification of mussel glues was long suspected to be related to this oxidation process. The double stickiness accounts for the strength of the bonding in natural conditions and also means that L-DOPA might be a suitable adhesive for attaching biological molecules to surfaces — and thus invaluable for medical applications.

Andrew Mitchinson

current is mediated by two nitrate ions transported from the cytoplasm into the vacuole, in exchange for each proton flowing from the vacuole to the cytoplasm (Fig. 1). This exchange (one proton for two anions) matches that found for the bacterial CLC-ec1 transporter¹.

Physiologically speaking, these findings mean that dissipation of the high proton concentration inside vacuoles (typical pH about 5.5) via AtCLCa can provide energy for packing nitrate into vacuoles. Consistent with this notion, nitrate concentrations in vacuoles can be 30–50-fold higher than in the cell's cytoplasm⁸. With a typical vacuolar membrane voltage of 130 mV (that is, +30 mV at the inner side of the vacuolar membrane), nitrate ion channels could function in the osmotic uptake of anions and the acidification of vacuoles. But these channels could not build up such a steep nitrate accumulation gradient in vacuoles. One possible hypothesis for the related mammalian CLC proton–anion exchangers is that mammalian endosomes and secretory vesicles may need to over-accumulate anions for proper cell function. De Angeli *et al.* also analysed the ion-selectivity of the vacuolar AtCLCa for different anions and found that nitrate and iodide are similarly permeable, whereas chloride is much less permeable. Maybe some bacterial or mammalian CLCs also show a preference for nitrate.

As far as structural aspects are concerned, the first ångström-resolution structure of a CLC protein to be determined was that of the bacterial proton–chloride exchanger CLC-ec1 (ref. 9). Remarkably, the structure of CLC chloride channels is probably very similar to that of the proton–chloride exchanger¹⁰. For example, the CLC channels form dimers, which include two independent anion-channel pores, with one pore in each CLC subunit (making them 'double-barrelled' channels)^{6,11}. The three-dimensional structure of the bacterial exchanger revealed a similar double-barrelled association of two CLC proteins⁹.

So, what is the main structural difference between a chloride channel and a proton–anion exchanger? This question will be kicked around for some time yet, but initial studies revealed two glutamic acid residues, E148 and E203, in CLC-ec1 that are crucial for proton coupling^{1–3,12}. Of these, E203 is an amino acid that is required for proton exchange and is found in all of the CLC exchangers studied thus far¹², including AtCLCa. With the classical separation of ion channels and ion exchangers melting away, would one dare to speculate that the same protein might be modified to act either as an ion channel or a proton exchanger?

De Angeli *et al.*⁴ find that of the seven CLC genes in the *Arabidopsis* genome, five encode the evolutionarily conserved glutamate residue at the position analogous to E203. It could be that the *Arabidopsis* CLC proteins are targeted to different cellular membranes^{4,13}, because plants use protons for various types of coupled

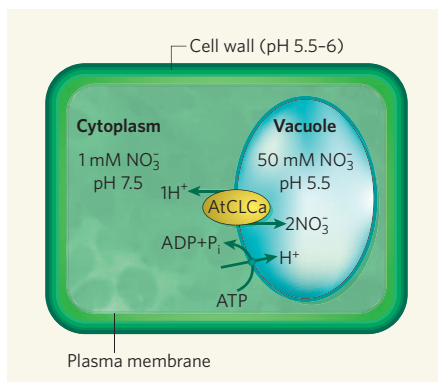


Figure 1 | Nitrate accumulation in plant vacuoles mediated by the proton–nitrate ($\text{H}^+ - \text{NO}_3^-$) exchanger AtCLCa. This process, revealed by De Angeli *et al.*⁴, provides a biochemical mechanism by which high concentrations of nitrate can be accumulated in vacuoles. The hydrolysis of cytoplasmic ATP to ADP provides the energy for the 'proton pump' that ushers protons from the cytoplasm into the vacuole, thus providing the energetic needs for AtCLCa function. (Modified from Fig. 4 of ref. 4.)

ion transport¹⁴. The plant plasma membrane faces an acidic extracellular pH (pH 5.5 to 6)¹⁴, and proton–anion exchange might also be involved in plasma-membrane transport. For example, rapid movements in plants are often mediated by a dramatic reduction in the ion content of 'motor' cells, such as those that control leaf and guard-cell movements. Plasma-membrane anion-selective channels control the efflux of ions from cells in these responses^{15–17}. However, as anions flow rapidly out of these cells, the anion concentration in the cell wall can increase, thus reducing the

electrochemical gradient for ion efflux. In this situation, proton–anion exchangers could boost anion efflux while also depolarizing the cell, which is required for parallel efflux of the positively charged counter-ion, potassium¹⁵.

For the future, two things seem certain. One is that study of CLCs will continue to revolutionize our understanding of the fast-shrinking differences between ion channels and ion exchangers. The other is that plant CLCs will provide excellent subjects for further illuminating the biology and the biophysical properties of these transporters.

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SOLID-STATE CHEMISTRY

Framework for a molecular prison

Michel Pouchard

A remarkable family of solids exists in which host compounds hold guest molecules captive in their rooms. The latest example has an unprecedented topology and opens up fresh avenues for investigation.

Small molecules can be trapped, like hostages in a prison cell, by networks of cages formed from another compound. The resulting solids — known as clathrates, or inclusion compounds — have been known about for more than 150 years, but have recently gained media attention for their potential to trap the greenhouse gas carbon dioxide. Many types of clathrate have been identified, and it seemed that little remained to be discovered. But in *Angewandte Chemie International Edition*¹, Karau and Schnick describe the synthesis of a clathrate with a unique cage structure.

Clathrates are formed when a host compound encloses guest molecules without using strong ionic interactions or covalent

bonds. This definition was proposed by the X-ray crystallographer Herbert Powell² in the 1940s, after he analysed the structure of the archetypal clathrate, β -hydroquinone. Powell found that solvent molecules are embedded in a hydrogen-bonded network of hydroquinone during crystallization. The name 'clathrate' was later applied to inclusion compounds with lattices constructed from covalent bonds, as exemplified by a series of silica (silicon dioxide) structures that are now known as clathrasils³. Nanoporous frameworks of silicon or germanium semiconductors⁴ and even some networks of inorganic covalent salt complexes⁵ have also been described as clathrates.

In true clathrates, a tetrahedral framework

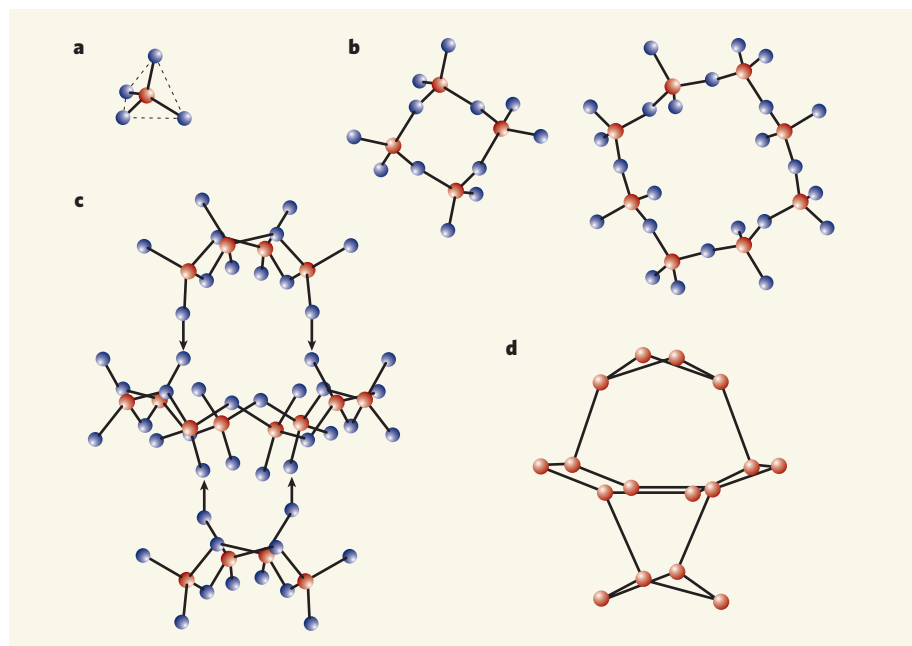


Figure 1 | Cage construction. The nitride clathrate reported by Karau and Schnick¹ consists of an atomic framework of cages. **a**, The building blocks of the framework are atomic tetrahedra, each made from a phosphorus atom (orange) surrounded by four nitrogen atoms (blue). **b**, The framework contains rings made from either four or eight tetrahedra, which share nitrogen atoms at the corners. **c**, Exploded side view of a cavity, formed from a ring of eight tetrahedra capped with two rings of four tetrahedra. The arrows point between nitrogen atoms that are shared by the central and capping rings. **d**, The framework of the resulting cage, showing only the tetrahedral centres (phosphorus atoms) for simplicity. The resulting cavity has a unique topology for clathrates, and would contain an ammonia molecule (not shown).

of hydrogen bonds creates voids in which guest species are trapped. A good example of this is methane hydrate, discovered deep in the oceans, in which methane molecules are embedded in a lattice of water. The resulting material looks like dirty ice, and could be a source of fossil fuel. Only extremely weak interactions are required for clathrates to hold guest molecules — for example, the gases xenon and krypton are renowned for being generally unreactive, but both can act as guests in inclusion compounds. The voids are often in the shape of symmetric polyhedra, such as a pentagonal dodecahedron.

Pioneering work³ in the 1960s on melanophlogite, a rare mineral form of silica with a clathrasil structure, led directly to the discovery of microporous materials⁶ in the 1990s. These materials contain a framework of pores that either has no electric charge, as represented by the formula APO_4 (where A can be boron, aluminium, gallium or indium, and P is phosphorus), or that is negatively charged, such as the well-characterized aluminosilicates $(\text{AlSiO}_4)^-$. The chargeless frameworks enclose neutral molecules, whereas the negatively charged systems host large, positively charged guests, such as the cations of the alkali metals rubidium and caesium. Microporous materials can be made with different pore sizes, but as the pore size increases, the cavities connect to form channels. Such channel-containing materials are known as zeolites.

Most classes of microporous material

contain frameworks of oxides, although a few examples exist that are based on chemically related sulphur and selenium compounds. Schnick and colleagues^{7,8} broke this mould by preparing the first microporous materials with nitrogen-containing frameworks, known as nitrides. The resulting anionic cages have very different chemical and physical interactions with their guests, compared with those of oxide frameworks. The first such compound to be made was a phosphorus nitride⁷ that contained small, negatively charged cages $(\text{P}_{12}\text{N}_{24})^{12-}$. Each cage had eight hexagonal faces and six square faces, and hosted cationic complexes made up of zinc and chlorine atoms. More recently⁸, the partial substitution of the oxygen atoms in oxide frameworks by nitrogen atoms yielded an analogous compound known as an oxonitridophosphate, which contained $(\text{P}_{12}\text{N}_8\text{O}_6)^{6-}$ cages enclosing lithium ions. This compound contained cages that were the same shape as those in the phosphorus nitride, but it could also be prepared as a zeolite-like framework, containing large channels⁹.

All of these nitride compounds were big discoveries, but they are not true clathrates as they contain charged host and guest species. Karau and Schnick¹ now describe the first synthesis of a true nitride–phosphate clathrate, which traps neutral ammonia molecules in small cages within an unprecedented atomic framework, $\text{P}_4\text{N}_4(\text{NH})_4$ (Fig. 1). The framework is constructed from tetrahedra; each of these consists of a phosphorus atom at the centre of

four nitrogen atoms, where the nitrogen atoms form the corners of the tetrahedron subunit. The tetrahedra share corners, so that each nitrogen atom acts as the corner for two adjacent subunits. To form a cage, eight of these tetrahedra form a ring, which is capped above and below by two smaller rings, each formed from four tetrahedra. The resulting cavity has a geometry previously unknown in clathrates. The overall framework consists of a succession of these cages arranged in a lattice.

To obtain this novel clathrate, the authors use high-pressure (11 GPa), high-temperature (600 °C) conditions to react two simple starting materials together — phosphorus nitride (P_3N_5) and ammonium azide (NH_4N_3). High-pressure conditions usually destabilize low-density materials, so it seems odd that a porous solid, such as a clathrate, can be made in this way. But in this case, the high pressure is required to prevent the thermal breakdown of the reagents — no mean feat, as ammonium azide is highly explosive at atmospheric pressure. Under the extreme reaction conditions¹, ammonium azide is converted into nitrogen and ammonia. The ammonia is incorporated into the growing nitride framework, and seems to act as a template to produce the unusual topology of the resulting clathrate.

The burgeoning interest in microporous materials is fuelled by their potential uses in catalysis, gas storage and separative membranes. The properties of Karau and Schnick's nitride clathrate¹ will undoubtedly be studied in detail, with a view to such exploitation. Their compound has remarkable thermal stability — no ammonia is released from the clathrate at temperatures up to 550 °C. This could be very useful, for example in membrane reactors, where the trapped molecules would take part in chemical reactions at solid–gas interfaces. Because the hydrogen atoms of the framework, in the form of protons, could be readily exchanged between the nitrogen atoms, the nitride may even exhibit electrical protonic conduction. But for chemists everywhere, this work reaffirms that there are areas of chemical space still left to be explored, and many fascinating compounds yet to be made. ■

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BRIEF COMMUNICATIONS

Kin preference in a social microbe

Given the right circumstances, even an amoeba chooses to be altruistic towards its relatives.

Kin recognition helps cooperation to evolve in many animals¹, but it is uncertain whether microorganisms can also use it to focus altruistic behaviour on relatives. Here we show that the social amoeba *Dictyostelium purpureum* prefers to form groups with its own kin in situations where some individuals die to assist others. By directing altruism towards kin, *D. purpureum* should generally avoid the costs of chimaerism^{2,3} experienced by the related *D. discoideum*.

Dictyostelium normally live as asexually reproducing, unicellular amoebae in forest soils. But when starved of their bacterial food source, they aggregate in thousands to form a multicellular, motile 'slug'. This eventually becomes a fruiting body^{4,5}, in which some amoebae in the group differentiate to form spores and other amoebae die to form a stalk structure that assists the dispersal of these spores. Stalk cells are therefore sacrificed to aid the others.

Because multicellularity in social amoebae is accomplished by aggregation of cells, fruiting bodies could consist of one or more clones. In the model organism *Dictyostelium discoideum*, genetically distinct clones can mix to form chimaeras, and one clone may sometimes exploit another by contributing less than its proportional share to the sterile stalk². However, tests with several clones of other species suggest that mixing may not be the rule^{6–8}.

We therefore tested for kin discrimination in *D. purpureum*. We performed 14 pairwise mixing experiments between isolates collected at different locations in the Houston Arboretum, Texas. One isolate of each pair was labelled with a fluorescent dye. Cells of the two isolates were plated in equal proportions at a density of about 2×10^6 cells per cm^2 and allowed to complete social development in the absence of bacteria. We then assessed the proportions of the two isolates in some 500 spores for each of 10 fruiting bodies.

Randomization tests revealed that 12 of 14 pairwise experiments showed evidence of strong kin discrimination: although different isolates aggregated together, individual fruiting bodies consisted of predominantly one isolate or the other, with significantly higher variances in the proportion of fluorescent spores than were found in randomly mixed clonal controls (Fig. 1a, and see supplementary information). Although two pairs of isolates did not discriminate in favour of kin, perhaps because of shared identity at one or more recognition alleles, kin

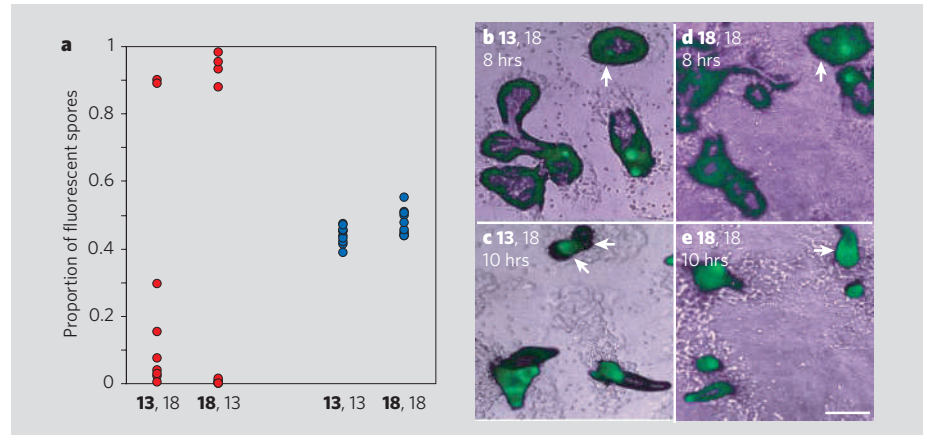


Figure 1 | Kin discrimination during social development in the amoeba *Dictyostelium purpureum*.

a, Scatter plot showing the proportion of fluorescently labelled spores in individual fruiting bodies formed at high amoeba density for isolates 13 and 18 (see text; bold, fluorescently labelled isolate). A greater variance in experimental (red circles) than in control treatments (blue circles) indicates clonal sorting ($P < 0.0002$). **b–e**, Fluorescent micrographs taken with the same field of view at different times of development at high density. **b**, Initial aggregates contain two isolates (13, labelled green; 18, unlabelled); **c**, these isolates then separate individually as 'slugs' that emerge from the aggregates. **d, e**, In controls of pure isolate 18, where half of the cells are fluorescently labelled, labelled and unlabelled cells mix equally at aggregation (**d**), and at the slug stage (**e**). Arrows designate individual aggregates (**b, d**) and emerging slugs (**c, e**). Scale bar, 1 mm.

discrimination was significant overall (Wilcoxon signed-rank test (WSRT), $Z = -3.111$, $n = 14$, $P = 0.0019$). The calculated relatedness in fruiting bodies from experimental mixes rose to 0.81, compared with the value of 0.50 expected for complete mixing.

We monitored the timing of kin recognition by using fluorescence microscopy (Fig. 1b–e). Different clones mixed during early aggregation (Fig. 1b) but separated when they emerged as slugs (Fig. 1c); however, single-clone controls remained mixed under the same conditions (Fig. 1d, e). These results indicate that kin discrimination is not due to differences in developmental timing between clones.

Strict exclusion of non-kin carries the risk of suboptimal group sizes when kin are rare⁹. To determine whether kin-discriminating clones of *D. purpureum* would mix with non-kin if kin were less abundant, we did 11 experiments at an amoeba density low enough to make fruiting bodies scarce (about 2×10^5 cells per cm^2). There was less sorting in these low-density experiments than in the high-density ones (WSRT, $Z = -2.667$, $n = 11$, $P = 0.0076$; see supplementary information). This effect was lost when high- and low-density experiments were done simultaneously in six pairwise mixtures (WSRT, $Z = -1.572$, $n = 6$, $P = 0.1159$);

however, the more important result is unambiguous. *Dictyostelium purpureum* preferentially associates with kin, and this remains true even at low density when partners are hard to find.

This kin discrimination means *D. purpureum* should avoid the disadvantages of forming chimaeras, and indeed only one clone was consistently cheated (see supplementary information). Our findings support the application of kin-selection theory to microorganisms and provide further evidence that social microbes can show sophisticated behaviour¹⁰ previously thought to occur only in higher organisms.

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GENE EXPRESSION

Long-term gene silencing by RNAi

Small RNA molecules participate in a variety of activities in the cell: in a process known as RNA interference (RNAi), double-stranded RNA triggers the degradation of messenger RNA that has a matching sequence; the small RNA intermediates of this process can also modify gene expression in the nucleus¹. Here we show that a single episode of RNAi in the nematode *Caenorhabditis elegans* can induce transcriptional silencing effects that are inherited indefinitely in the absence of the original trigger. Our findings may prove useful in the ongoing development of RNAi to treat disease.

It has been shown that phenotypes induced in *C. elegans* by RNAi can last for two or three generations². Because the generation time of a worm is only three days, however, it is not clear whether this effect can be explained simply by a slow dilution of the silencing factors.

We have therefore investigated the heritability of gene silencing by RNAi over many generations in *C. elegans* and used an RNAi screen to identify genes that may influence this inheritance. (For details of methods, see supplementary information.)

We injected wild-type Bristol N2 worms with a double-stranded RNA that targets the *C. elegans* gene *ceh-13* for one generation. The *Ceh-13* phenotype, in which the worm is small and dumpy, persisted in some animals indefinitely. Inheritance was not fully penetrant: only about 30% of the progeny of *Ceh-13* worms inherited the phenotype. Wild-type siblings never had progeny with the *Ceh-13* phenotype, and crossing worms that had a *Ceh-13* phenotype with unaffected males showed that the trait is dominant. A single episode of RNAi can therefore induce heritable

silencing that is not fully penetrant and behaves in a dominant fashion.

To show that this is a general phenomenon, we targeted 171 other genes and found 13 that could be inheritably silenced (among them were *dpy-6*, *dpy-28* and *unc-73*; data not shown).

We also showed that a single transgenic copy of a gene (*gfp*) expressing green fluorescent protein (GFP) could be silenced, and the silencing inherited. We used animals expressing GFP under the control of a germline-specific promoter and created interference by feeding them bacteria that express double-stranded RNA homologous to *gfp*; progeny that did not express GFP were then transferred to new plates. In all siblings, GFP expression was reduced relative

to wild-type expression (Fig. 1). We detected animals that had reduced GFP expression over 80 generations.

Is RNAi the mechanism behind the initial silencing? There are two features of effective interference in *C. elegans*: it targets exons, not introns, and it depends on the canonical RNAi genes *rde-1* and *rde-4* (ref. 3) (see supplementary information). Tests for both show that RNAi is responsible for the effect, and this is further supported by our observation that genes are more likely to be indefinitely silenced in worms with a mutation in *eri-1* (results not shown), which are hypersensitive to RNAi (ref. 4).

But RNAi probably does not underlie the inheritance mechanism — *rde-1* and *rde-4* are dispensable. To investigate further, we used a candidate RNAi screen to identify genes that affect the maintenance of silencing and found four that abolish inheritance when knocked out: *hda-4* (a class II histone deacetylase), *K03D10.3* (a histone acetyltransferase of the MYST family), *isw-1* (a homologue of the yeast chromatin-remodelling ATPase ISW1) and *mrg-1* (a chromo-domain protein) (see supplementary information).

The fact that these genes are all involved in chromatin remodelling suggests that the inheritance of RNAi-induced phenotypes is due to silencing at the transcriptional level. It may be that this is achieved by modification of specific residues in histone tails, because culturing worms in the presence of the histone deacetylase inhibitor trichostatin A relieves silencing.

Earlier work has revealed a link between RNAi and transcriptional silencing⁵ and inheritance of silencing for one generation in mice⁶. We have shown that RNAi can induce silencing effects that, once established, are inherited indefinitely over generations of sexual reproduction, in the absence of the trigger and of RNAi machinery.

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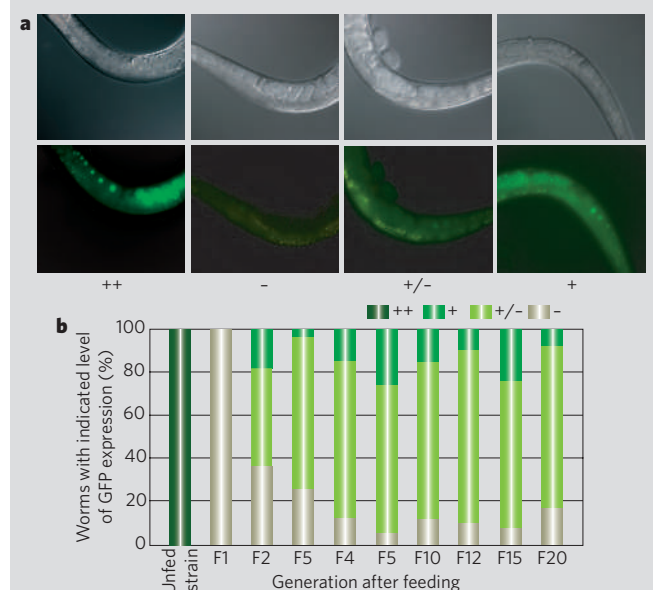


Figure 1 | RNA interference triggers inheritable silencing of a transgene encoding green fluorescent protein (GFP). **a**, Four degrees of GFP expression in *Caenorhabditis elegans* are revealed using Nomarski optics (top panels) and ultraviolet illumination (bottom panels): ++, very bright, only observed in untreated transgenic NL3630 worms; -, +/– and +, weaker GFP signals from progeny of an RNAi-treated worm that did not express GFP. **b**, Worms were fed on bacteria expressing double-stranded RNA targeting the transgene *gfp*. Ten independent lines were quantified for 20 generations. Shown is the mean percentage of worms with the indicated amount of GFP expression in the progeny of a worm that did not express GFP.

PROTEIN EVOLUTION

Causes of trends in amino-acid gain and loss

Arising from: I. K. Jordan *et al.* *Nature* **433**, 633–638 (2005)

Understanding how proteins evolve is important for determining the molecular basis of adaptation, for inferring phylogenies and for engineering novel proteins. It has been suggested that some amino acids were incorporated into the genetic code more recently than others¹ and, after comparing pairs of closely related genomes, Jordan *et al.*² report that 'recent' amino acids are becoming more common. They argue that this process has been going on since the genetic code first evolved to encompass all 20 amino acids. Here we provide evidence that the patterns observed conform with standard, nearly neutral theoretical

expectations³ and require no new explanation. This reinforces the need for caution in the interpretation of results derived from closely related taxa.

If all changes are neutral, then the equilibrium abundance of amino acids is dictated by mutation alone. At mutational equilibrium, the number of mutations causing loss of a given amino acid will equal the number of gains. But if selection skews amino-acid use, mutation will typically generate more of those amino acids that are under-represented, with this excess being selectively purged³. There is, however, a time lag between the mutational moderation by selection⁴: evidence for this can be found by examining the ratio of the number of synonymous substitutions per synonymous site to the number of non-synonymous substitutions per non-synonymous site, dS/dN. This ratio increases as a function of the time since common ancestry between two genomes⁴ and it may explain apparently accelerated rates of short-term evolution^{5–7}. Jordan *et al.*² notably used closely related genomes for their analysis, so might their observed trends of gain or loss of amino acids simply reflect mutation before moderation by purifying selection³? We provide several tests of this possibility and fail to falsify it.

First, we compared the observed profile of amino-acid gain (or loss) with that expected under mutation alone, using nucleotide changes in intergenic DNA as the mutational bench-

mark. We examined three bacterial groups (*Staphylococcus*, *Bacillus* and *Escherichia/Shigella*), considering one focal lineage in each case. We applied, through simulation, the lineage-specific mutational profile to the respective codon frequencies until the appropriate non-synonymous distance (Fig. 1, methods), and calculated the mean normalized gain–loss ratio for each amino acid. As the mutational model predicts, the simulated and observed patterns of gain and loss are similar (Fig. 1).

The mutational model also predicts that gained amino acids should occur at frequencies below mutational equilibrium. From the intergenic mutations, we approximated the equilibrium frequency of each codon as the product of estimated equilibrium levels of its nucleotides. As expected, gainers are underused (correlation between the observed normalized gain/loss ratio and mean per-codon deviation from mutational equilibrium: *Staphylococcus*: $R = -0.74$, $P = 0.00021$; *Bacillus*: $R = -0.53$, $P = 0.016$; *Escherichia*: $R = -0.50$, $P = 0.027$). As the putatively new amino acids are also those that are underused^{2,8} (rank correlation between putative recruitment order² and per-codon deviation from equilibrium: *Staphylococcus*: $R = -0.667$, $P = 0.0013$; *Bacillus*: $R = -0.46$, $P = 0.04$; *Escherichia*: $R = -0.603$, $P = 0.0048$), mutation alone should preferentially generate the newer amino acids.

If purifying selection is moderating the mutation bias, gain–loss bias should decay

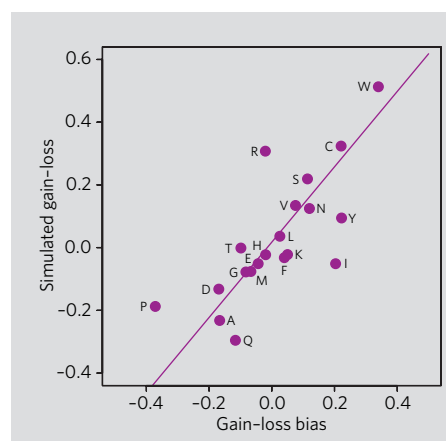


Figure 1 | The observed profile of gains or losses per amino acid compared with that expected from the underlying nucleotide-level mutation bias and codon content. Data are from the focal *Staphylococcus* lineage; line indicates the principal axis regression line. Pearson product moment correlation: $R = 0.755$, $P = 0.00012$. For lineage *Bacillus*: $R = 0.502$, $P = 0.023$; for *Escherichia*, $R = 0.73$, $P = 0.0003$.

Methods. To estimate mutational profiles of the focal lineages, we compared intergenic sequence of each focal genome aligned with orthologous sequence from other genomes available for the same taxon (*Staphylococcus*, $n = 6$; *Bacillus*, $n = 5$; *Escherichia coli* / *Shigella*, $n = 5$). Only differences unique to the focal lineages were considered. The number of each of the 12 possible nucleotide changes was then divided by the frequency of the ancestral nucleotide and normalized. In the simulations, we randomly picked a codon according to its relative frequency in the focal genome, and randomly picked a base in the codon. We then mutated this base according to the mutational profile frequencies. We reiterated until the total number of non-synonymous changes reached the observed number for the focal lineage. By comparison with the initial codons, we then determined the number of gains (g) and losses (l) of each amino acid and calculated the normalized bias: $(g - l)/(g + l)$. The simulation was repeated 1,000 times.

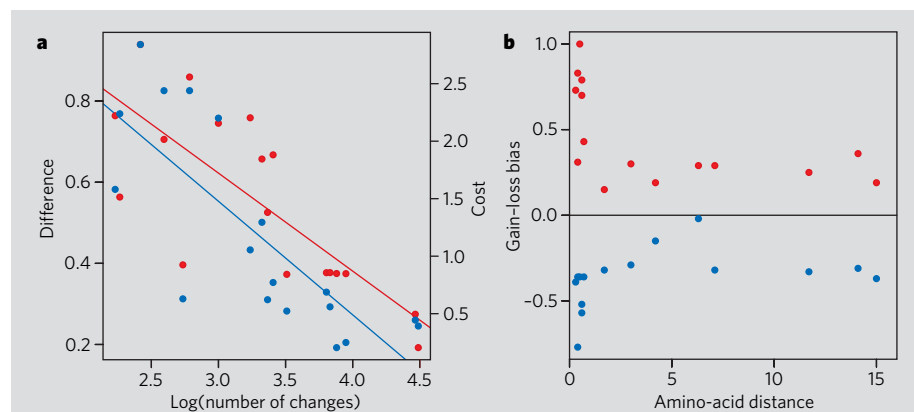


Figure 2 | Relation between divergence between taxa and bias in amino-acid gain and loss. **a**, The difference between the normalized mean gains and losses in the strongest gainers and the strongest losers (blue) and the average cost of each amino-acid replacement (red) as a function of the log of the number of non-synonymous changes in the relevant comparisons. When the comparator species are more divergent, there is a significant decrease in the net gain or loss (blue line: $R^2 = 0.629$, $P < 0.0005$). There is a significant decrease in the average cost of each amino-acid replacement as the sequences diverge (red line: $R^2 = 0.601$, $P < 0.0005$). **b**, The gain/loss profile for proline (strong loser, blue) and cysteine (strong gainer, red) as a function of amino-acid distance in 14 diverse taxa (data from Table 1 of ref. 2). The bias is strongest in those cases in which the amino-acid distance is short and there has been little time for purifying selection. Further details are available from the authors (<http://www.bath.ac.uk/bio-sci/hurst.htm>).

over time³. Using the multiple genomes for each of our three taxa, we compared biases with the degree of divergence. We computed the average normalized gain/loss ratio for consistent gainers (S, C, F, I and V, in single-letter amino-acid notation) and consistent losers (A, D, G, P and Q), and the mean difference between these averages. As predicted, the magnitude of these biases decreases with increasing divergence over time (Fig. 2a). A diminution of bias with time is also seen in the data of Jordan *et al.* (Fig. 2b). Note that the bias need never equal zero as polymorphism will always be present.

The underuse of new amino acids may reflect their greater expense (correlation of cost⁹ with putative² order of recruitment: $R^2=0.67$, $P<0.0001$), as biosynthetically cheaper amino acids are preferred⁹ (correlation between deviation away from mutational equilibrium per codon and cost of amino acid: *Staphylococcus*: $R = -0.61$, $P = 0.004$; *Bacillus*: $R = -0.43$, $P = 0.058$; *Escherichia*: $R = -0.57$, $P = 0.008$).

Serine aside, the amino acids that are consistently gained are more expensive than those consistently lost. And there is indeed a decrease in the average cost per replacement as more diverged sequences are compared (Fig. 2a).

In sum, as expected under the nearly neutral model, mutation is biased towards the newer or costlier amino acids, but time-lagged fixation is biased towards older or cheaper amino-acid replacements. The expectation² of long-term bias is an artefact of extrapolation from short-term changes, and this highlights the need for caution in analyses of closely related taxa. A real bias may, however, sometimes be observed. When a lineage moves closer to the mutational equilibrium, a shift towards newer or more expensive amino acids is expected. Humans could be an example, as we may be accumulating deleterious mutations owing to reduced population size¹⁰.

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PROTEIN EVOLUTION

Jordan et al. reply

Replying to: L. D. Hurst, E. J. Feil & E. P. C. Rocha *Nature* **442**, doi:10.1038/nature05137 (2006)

Hurst *et al.*¹ and, earlier, McDonald² confirm the pattern of amino-acid gain and loss that we report³. However, they attribute this pattern to properties of the mutation-selection equilibrium, arguing that gainer amino acids are more common than losers among weakly deleterious, rare polymorphisms, which segregate within one or both compared species but never reach fixation. Indeed, we all^{1–3} concur that gainers are, mostly, under-represented, whereas losers are over-represented with respect to mutations (Table 3 of ref. 3). Still, we cannot agree that the effect of weak negative selection is a viable alternative to our original explanation.

Hurst *et al.* show that the observed pattern is similar to that expected from the mutation process¹. This is inconsistent with their own hypothesis in that the observed trend would be significantly weaker than the mutational bias owing to the effect of negative selection against deleterious mutations.

As we³ and Hurst *et al.*¹ have shown, up to 30% of all substitutions contribute to the observed pattern for strong gainers and losers, even in relatively distant species at about 15% amino-acid divergence. Under the hypothesis of Hurst *et al.*, this would require about 4.5% of amino-acid sites in a genome to be occupied by deleterious alleles contributing to gain and loss. This is an order of magnitude greater than the divergence between many analysed bacterial

species (Table 1 of ref. 3). Therefore, the trend cannot be explained according to Hurst *et al.*, even under the unrealistic assumptions that all deleterious polymorphism contributes to gain and loss, that all amino-acid polymorphism is deleterious, and that the divergence between bacterial species is due entirely to within-population polymorphism.

Direct comparison of amino-acid polymorphism and divergence can clarify whether the presence of non-fixed amino-acid variants is the main cause of the observed pattern. At present, comprehensive single-nucleotide-polymorphism data are available only for *Homo sapiens*. Although purifying selection is relaxed in humans compared with most other species^{4,5}, more than 10% of human amino-acid alleles are deleterious^{5–8}. Thus, the human data can be used to analyse the effect of deleterious polymorphism on the trend. Only 2.6% of the non-synonymous differences between the public human genome and the chimpanzee genome coincide with the differences between the public genome and the Celera individual-A genome sequence. Given a similar polymorphism level in the chimpanzee, about 5% of the human–chimpanzee divergence is expected to be due to non-fixed mutations. Thus, even if all polymorphism resulted from segregation of rare deleterious gainer alleles, the observed pattern, which substantially exceeds 5% for eight out of nine

strong gainers and losers, cannot be explained by polymorphism.

If mutations producing gainers and mutations eliminating losers were deleterious, levels of gain and loss in polymorphism would be substantially higher than in divergence, and the shift in allele frequency distribution would be observed in corresponding single nucleotide polymorphisms. However, the data on human single nucleotide polymorphisms do not conform to this expectation (see, for example, Table 2 of ref. 3).

We conclude that mutation-selection equilibrium is not an acceptable explanation of the universal trend in amino-acid gain and loss.

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RecA acts in *trans* to allow replication of damaged DNA by DNA polymerase V

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The DNA polymerase V (pol V) and RecA proteins are essential components of a mutagenic translesion synthesis pathway in *Escherichia coli* designed to cope with DNA damage. Previously, it has been assumed that RecA binds to the DNA template strand being copied. Here we show, however, that pol-V-catalysed translesion synthesis, in the presence or absence of the β -processivity-clamp, occurs only when RecA nucleoprotein filaments assemble or RecA protomers bind on separate single-stranded (ss)DNA molecules in *trans*. A 3'-proximal RecA filament end on *trans* DNA is essential for stimulation; however, synthesis is strengthened by further pol V–RecA interactions occurring elsewhere along a *trans* nucleoprotein filament. We suggest that *trans*-stimulation of pol V by RecA bound to ssDNA reflects a distinctive regulatory mechanism of mutation that resolves the paradox of RecA filaments assembled in *cis* on a damaged template strand obstructing translesion DNA synthesis despite the absolute requirement of RecA for SOS mutagenesis.

In 1974, Miroslav Radman proposed that *E. coli* possessed an inducible response to protect cells from the deleterious consequences of DNA damage¹. The hypothesis was elaborated on shortly thereafter by Evelyn Witkin², and the “SOS response” (as it has come to be known) has been well characterized in the ensuing three decades and is now recognized to involve the upregulation of over 40 proteins engaged in DNA repair, replication and recombination under the control of the LexA repressor³. It has tacitly been assumed that uncoupling of leading and lagging strand replication at blocking lesions results in unwound regions of ssDNA ahead of the stalled replication fork, which serve as a platform for RecA nucleoprotein filament formation⁴. If SOS-induced non-mutagenic repair processes are insufficient to reactivate cellular DNA replication, a mutagenic phase of SOS is initiated, facilitated largely by the translesion polymerase, pol V (also known as the UmuD₂C complex)⁴. Translesion DNA synthesis (TLS) is generally successful in restarting DNA replication, and is accompanied by a large (~100-fold) increase in mutations targeted principally at sites of DNA damage³.

Both *in vitro*^{5–8} and *in vivo*^{9–11}, pol V activity is almost entirely dependent on the RecA protein. A RecA filament formed on damaged DNA located *cis* to pol V has thus been the focus of previous studies on the molecular mechanisms of pol-V-dependent TLS^{8,12,13}. Indeed, the role of RecA in TLS has progressed through several iterations, from simply positioning UmuD₂C at the template or primer^{14,15}, to a moving interaction that changes as the RecA filament is displaced by pol V¹², and, most recently, as an integral component of pol V¹⁶ (Supplementary Fig. 1). However, one crucial factor was overlooked in all of these studies—the possibility that RecA might act in *trans*, rather than in *cis*, to stimulate pol V.

Transactivation of pol V by RecA bound to ssDNA

Linear and closed-circular primer/template DNA contains either excess primer or template strands. The use of hairpin DNA instead of primer/template DNA ensures the absence of unannealed ssDNA. Pol V alone catalyses weak primer elongation on hairpin DNA containing a 3-nucleotide-long template overhang (Fig. 1a, lane 1;

Supplementary Fig. 2). RecA in the presence of ATP does not stimulate pol V (Fig. 1a, lane 2). However, the addition of a non-homologous ssDNA (*trans* 80-mer) activates RecA for robust primer extension by pol V (Fig. 1a, lane 3). These data demonstrate that strong stimulation of pol V occurs when RecA is activated by a *trans* DNA molecule—that is, one that is not copied by pol V. (Supportive control data are provided in Supplementary Fig. 3.)

We examined whether transactivation is required for pol V activity when the β -processivity-clamp is included in the reaction (Fig. 1b). To ensure efficient β -clamp loading by the γ -clamp-loading complex, a hairpin with a 12-nucleotide overhang was used, and a biotin–streptavidin barrier was attached to prevent the β -clamp from sliding off. Processivity of another SOS DNA polymerase, pol IV, is enhanced (Fig. 1b, lane 5), verifying that the clamp has been loaded properly. However, pol V is barely active in the presence of the β -clamp (Fig. 1b, lane 1). RecA inhibits pol-IV-mediated synthesis in the absence or presence of the β -clamp (Fig. 1b, lanes 6 and 7, respectively), indicating that RecA is able to interact with the hairpin DNA. Nonetheless, RecA is incapable of stimulating pol V (Fig. 1b, lane 2), unless *trans* ssDNA is also present (Fig. 1b, lane 3).

Pol V typically copies past DNA lesions—for example, abasic sites (“X” in Fig. 1c–e). However, it is only able to perform TLS when not only RecA, but also ssDNA in *trans*, is present in the reaction (Fig. 1c, lanes 3 and 5). TLS is measured as the fraction of primers extended past the lesion and is enhanced by approximately threefold from 7% to 22% in the presence of the β -clamp (Fig. 1c, lanes 3 and 5). The enhancement of lesion bypass requires the presence of streptavidin-bound DNA to prevent dissociation of the β -clamp (Fig. 1c, lane 6). These data indicate that transactivation by RecA is necessary for both normal and translesion synthesis in the presence or absence of the β -clamp.

It has been a mechanistic question whether or not *bona fide* RecA filaments were required for pol V TLS^{8,13} or whether smaller RecA–DNA complexes could suffice^{16,17}. We extended the template overhang to 50 nucleotides to determine whether transactivation is still required when RecA filaments are formed in *cis* (Fig. 1d). As observed

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with the shorter overhangs, pol-V-catalysed primer elongation (Supplementary Fig. 4) and TLS (Fig. 1d) is essentially inactive except when RecA and ssDNA are provided *in trans*. We verified that hairpin DNA containing a 50-nucleotide overhang stimulates autocleavage of LexA, showing that RecA, which can act as a co-protease, is not excluded from the *cis* DNA (Supplementary Fig. 4).

DNA lesions can occur in the leading strand, modelled by the primer/template hairpin DNA in Fig. 1d. *In vivo*, however, pol V could also act on damaged lagging strand gaps. To address the possibility of differential activation requirements on gapped DNA substrate, we confirmed the requirement for transactivation on double hairpins with or without lesions (Supplementary Fig. 5).

Primed ssDNA circles have been used previously to characterize the effects of pol-V-catalysed TLS^{7,8,18,19}. To ensure that our observations are not an anomaly related to the use of hairpin DNA, a 30-mer primer was annealed to a synthetic 240-mer circle in 1:1

stoichiometry. As observed for the hairpin substrates, pol V is barely active in the presence of RecA, the β -clamp or both (Fig. 1e, lanes 1–4; Supplementary Fig. 6). However, robust synthesis and TLS are observed when unprimed single-stranded circular DNA mimicking the presence of excess unannealed circular template is provided in *trans*, with processivity enhanced by the β -clamp (Fig. 1e, lanes 5 and 6; Supplementary Fig. 6). Thus, the addition of separate transactivating ssDNA molecules seems to be a stringent requirement for RecA-mediated stimulation of pol V synthesis on damaged and undamaged DNA templates (Fig. 1).

In vivo data indicate that gaps persist and disappear much later during the SOS response²⁰, so the majority of RecA–ssDNA complexes in cells induced for SOS could occur within gapped DNA. Therefore, in comparison with transactivating single-stranded linear DNA, hairpin DNA and single-stranded circular DNA, we determined that RecA bound to ssDNA gaps also transactivates pol V

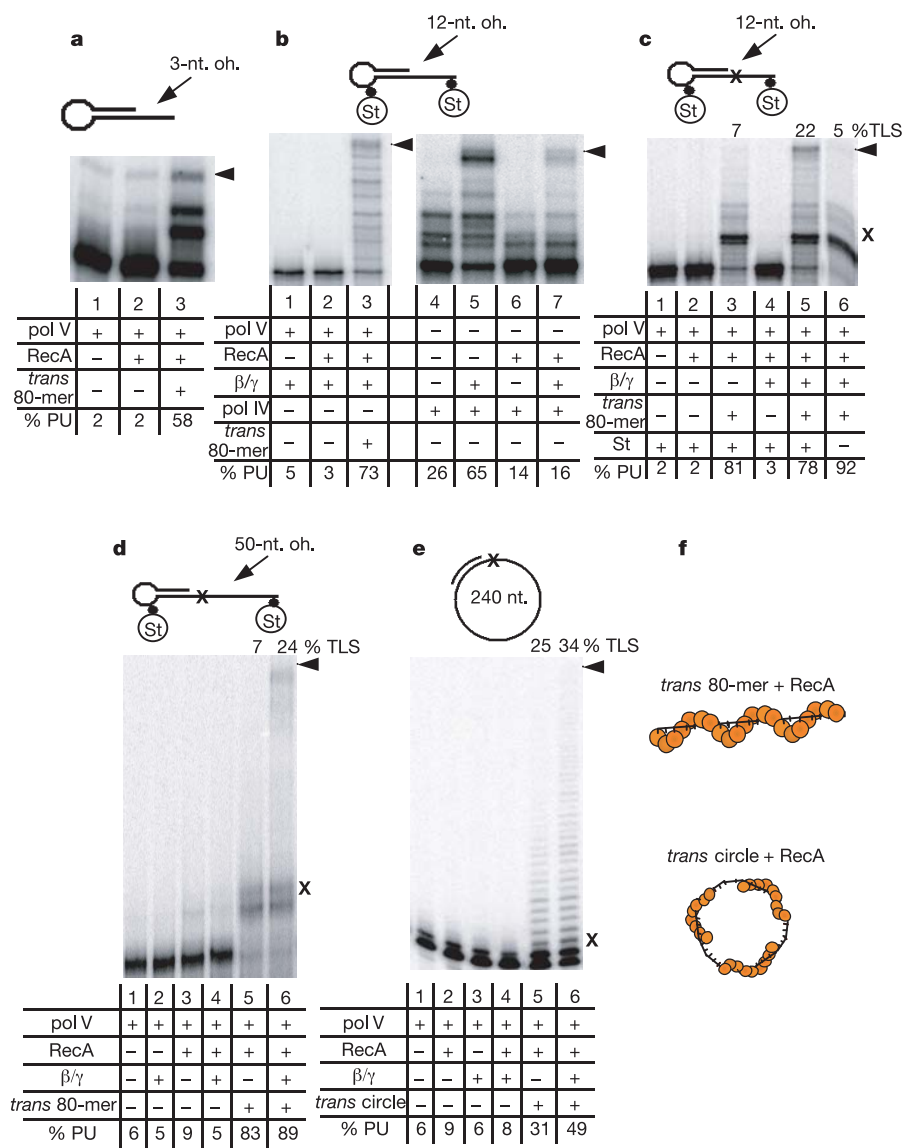


Figure 1 | RecA–ssDNA transactivation of pol V on damaged and undamaged DNA templates. **a**, Transactivation of pol-V-catalysed synthesis on a hairpin DNA with a 3-nucleotide (nt.)-long template overhang (oh.) by a ssDNA (80-mer)–RecA complex present in *trans* (lane 3). **b**, RecA–DNA transactivation of pol V in the presence of the β/γ -sliding-clamp and clamp-loading complex (left gel); control reactions with pol IV (5 nM) (right gel). St denotes the presence of streptavidin–biotin blocks. **c**, Transactivation of pol-V-catalysed TLS. X denotes the site of the DNA

lesion (an abasic site). **d**, Transactivation of pol V synthesis on a DNA hairpin containing a 50-nucleotide-long template overhang with a lesion, X. **e**, Transactivation of pol V on damaged (X) circular DNA by RecA bound to *trans* circular DNA. **f**, Sketch of transactivating DNA. In **a–e**, the arrowheads indicate the position of the full-length product; % PU is the percentage of hairpin templates extended (primer utilization); transactivating DNA molecules are at 160 nM, when present; and RecA is at 4 μ M, when present. In **c–e**, % TLS is the percentage of primers extended past the lesion, X.

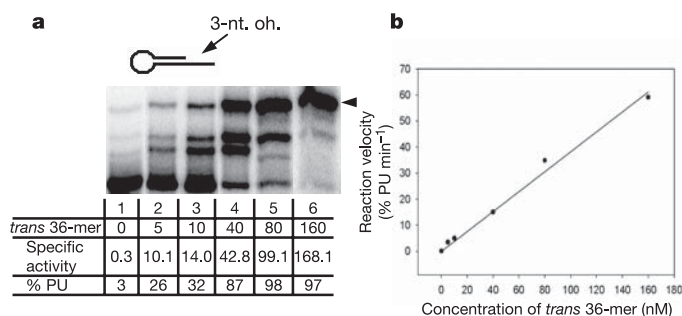


Figure 2 | Kinetics of pol V transactivation by RecA-ssDNA. **a**, Pol-V-catalysed primer utilization when copying a hairpin containing a 3-nucleotide-long template overhang was measured in the presence of ATP- γ S and RecA (2 μ M) at varying *trans* ssDNA (36-mer) concentrations (in nM). The specific activity of pol V is measured in pmol DNA per μ g pol V per min. The arrowhead indicates the position of the full-length product. **b**, Primer extension velocity depends on *trans* DNA concentration. The kinetic data used to calculate the reaction velocity is shown in Supplementary Fig. 9.

(Supplementary Fig. 7). The degree of transactivation is greater for longer ssDNA regions. These data show that *trans*-stimulation of pol V occurs when RecA binds to linear ssDNA (Fig. 1a–f), or any form of gapped DNA (Supplementary Fig. 7).

Transactivation kinetics of pol V by RecA bound to ssDNA

To facilitate the characterization of the transactivation kinetics, reactions were performed using the slowly hydrolysable ATP- γ S

(Fig. 2), which inhibits filament disassembly. Synthesis was measured on 3-nucleotide-overhang hairpin DNA substrates to eliminate inhibition by the *cis* RecA filaments that can assemble only on longer ssDNA overhangs (Supplementary Fig. 8). A linear relationship is observed between the velocity of pol V synthesis and the concentration of *trans*-DNA–RecA complex. The second-order dependence of pol V activity on the *trans*-DNA–RecA complex is evidence of a bimolecular transactivation reaction (Fig. 2b). The specific activity of pol V increases 30-fold in the presence of 5 nM *trans* ssDNA, which is substoichiometric to the hairpin DNA (Fig. 2a, lane 2), compared with pol V in the absence of *trans* DNA (Fig. 2a, lane 1). A fourfold excess of *trans* ssDNA (80 nM) over hairpin DNA (20 nM) results in a 400-fold increase in the specific activity of pol V (Fig. 2a, lane 5). An eightfold excess of *trans* (160 nM) over hairpin (20 nM) DNA results in complete primer utilization within 20 min (Fig. 2, lane 6), and even within 5 min (Supplementary Fig. 9).

Pol V transactivation requires a free 3' RecA filament end

The 3'-proximal end of the RecA nucleoprotein filament on *trans* ssDNA has a crucial stimulatory role (Fig. 3). A single-stranded 30-mer was biotinylated at the 5' end and attached to a streptavidin-coated magnetic bead (Supplementary Fig. 10). The *trans*-activator bead strongly stimulates pol V activity (Fig. 3a, lane 2). When the biotin tag is switched to the 3' end of the DNA, RecA will nucleate on the DNA–bead complexes with the same 5'-to-3' directionality. In this case, however, the bead blocks access to the 3'-proximal end. This inverted filament is substantially less effective in stimulating pol V (Fig. 3a, lane 3), even though comparable amounts of RecA are bound to each of the DNA–bead complexes (Supplementary Fig. 11).

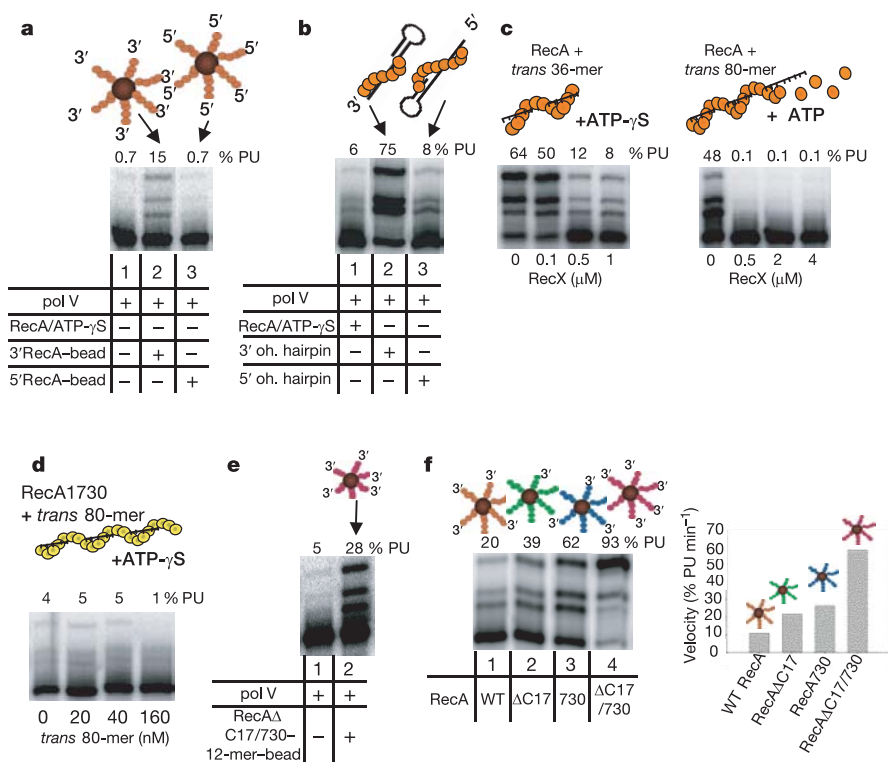


Figure 3 | A RecA filament end oriented 3' on *trans* DNA is required for efficient stimulation of pol V. **a**, RecA protein ($\sim$ 4 μ M) immobilized on a DNA–bead complex ($\sim$ 1 μ M of DNA molecules bound to the bead) with exposed 3'-proximal RecA ends causes strong transactivation of pol V. A filament with exposed 5' ends shows weak transactivation. **b**, RecA (2 μ M) bound to a hairpin DNA containing a 3' overhang stimulates pol V much more than one with a 5' overhang. **c**, RecX protein inhibits pol V synthesis in the presence of RecA (1 μ M), *trans* 36-mer (80 nM) and ATP- γ S (left panel), or RecA (4 μ M), *trans* 80-mer (160 nM) and ATP (right panel). **d**, Mutant

RecA1730 (4 μ M) in the presence of ATP- γ S and *trans* 80-mer (160 nM) is deficient in transactivation. **e**, Mutant RecA Δ C17/730(E38K) ($\sim$ 1 μ M) immobilized on a 12-mer-DNA–bead complex ($\sim$ 1 μ M of DNA molecules bound to the bead) causes robust transactivation. **f**, Wild-type (WT) and mutant RecA proteins ($\sim$ 4 μ M) immobilized on a 30-nucleotide-DNA–bead complex stimulate pol V synthesis with significantly different efficiencies. Hairpin primer/template DNA contains a 3-nucleotide-long template overhang (see diagram in Fig. 2a). (See Supplementary Methods and Supplementary Fig. 10 for preparation of RecA–DNA bead complexes.)

The requirement for an accessible 3'-proximal RecA filament end on ssDNA for pol V stimulation was confirmed by using hairpin oligonucleotides with 3' or 5' overhangs as the *trans*-activator DNA (Fig. 3b). Presumably, RecA filamentation on hairpins with a 5' overhang continues into the double-stranded regions, therefore excluding a 3' end on the ssDNA. The regulatory protein²¹ RecX blocks RecA filament extension by binding 3' to filament ends²². RecX also inactivates RecA transactivation of pol V, most likely via a steric block, even when RecA is formed on the DNA with the slowly hydrolysable ATP- γ S before the addition of RecX (Fig. 3c, left panel). RecX is even more inhibitory in the presence of ATP (Fig. 3c, right panel). The RecA1730 mutant protein confers an SOS non-mutable phenotype^{10,23}, and is unable to stimulate pol V activity *in vitro*¹⁶. The same holds true for the transactivation reactions (Fig. 3d). In the RecA1730 mutant, a phenylalanine replaces a serine at amino-acid residue 117, a position that is located at the surface facing 3' on a RecA filament end¹⁵.

Hierarchy of pol V transactivation by RecA mutants

Mutant RecA Δ C17/730(E38K)²⁴ associates tightly with ssDNA¹⁶ and is able to transactivate pol V while attached to a 12-mer-DNA-bead complex (Fig. 3e, lane 2). RecA-12-mer-DNA complexes do not provide sufficient space for a complete helical turn of a RecA filament. Notably, owing to the presence of the beads, these short complexes cannot stack to form longer filaments. Thus, the data support the idea that no more than a small RecA protomer is necessary for transactivation. Nevertheless, a longer *trans* ssDNA oligomer (30 nucleotides) increases RecA stimulatory activity (Fig. 3f, left panel, lane 4).

The three mutant RecA proteins RecA Δ C17²⁴, RecA730(E38K)²⁵ and RecA Δ C17/730(E38K)¹⁶ all bind tighter to DNA than wild-type RecA, and for that reason were believed to enhance RecA function. However, RecA binding properties are irrelevant in experiments with magnetic beads because comparable amounts of RecA protein are stably bound to the DNA-bead complex (Supplementary Fig. 11). The various RecA proteins show a hierarchy in pol V stimulation (Fig. 3f). Mutant RecA Δ C17 is missing its carboxy-terminal lobe, which otherwise occupies parts of the helical groove in a RecA filament²⁶. Therefore, this deletion may facilitate access to the groove for proteins such as UmuD', as has been modelled previously²⁷. RecA Δ C17 doubles the reaction velocity (Fig. 3f, right panel) and primer utilization by pol V (Fig. 3f, lane 2) compared with wild-type RecA. It is therefore tempting to speculate that the additional stimulation seen in the presence of RecA Δ C17 is attributable to a facilitated interaction between UmuD' and the helical groove of a RecA filament, as suggested in a previous study using electron microscopy²⁷.

Discussion

Here we propose a model for the role of pol V in SOS DNA-damage-induced mutagenesis in which an interaction between pol V and the surface of a RecA protomer or 3'-proximal RecA filament end is both necessary and sufficient for lesion bypass (Fig. 4a, orange-red circle). On the basis of *in vivo* studies with RecA mutants, Devoret and co-workers^{15,28,29} proposed that a 3'-proximal RecA filament end interacting with UmuD'C (later identified as pol V (UmuD'₂C)⁴) at a site of DNA damage in *cis* was necessary for TLS. Conversely, the central conclusion of the transactivation model is that interactions between pol V and RecA responsible for synthesis stimulation must occur while the proteins are bound to different molecules of DNA. This effect was obscured in previous studies investigating pol-V-catalysed TLS from different groups^{7,8,13,18,19,30}, including ours^{5,6,12,16,17}, where some fraction of the ssDNA could always have bound RecA capable of activating pol V complexes on other primer/template molecules *in trans*.

The activity of pol V increases linearly with the concentration of ssDNA supplied in *trans* (Fig. 2b). The bimolecular nature of the

reaction represents strong kinetic evidence that stimulation of pol V by RecA *per se* is dependent on a second *trans* DNA molecule bound to RecA. Complete hairpin DNA extension is observed on DNA substrates that preclude RecA inhibition in *cis* (Fig. 2a). The presence or absence of the β -clamp does not alter the requirement that RecA bound to DNA in *trans* is required for pol-V-catalysed TLS (Fig. 1).

RecA nucleation on ssDNA can occur at any segment, and is followed by filament extension in the 3' direction³¹ while disassembly, driven by ATP hydrolysis, occurs with the same directionality, thus creating new nucleation sites. Therefore, free 3'-proximal RecA filament ends will continuously be generated on ssDNA, such as (for example) circular DNA (Fig. 1e) or gapped DNA (Supplementary Fig. 7), and can serve as transactivation docks for pol V. Presumably, the longer the single-stranded region stretches, the more RecA filaments are able to initiate, thereby increasing the number of available 3'-proximal ends. Notably, a gapped circle containing longer ssDNA (Supplementary Fig. 6) or an unprimed single-stranded 240-mer circle (Fig. 1e, lane 5) provided in *trans* increases the extent of pol V stimulation.

An unambiguous consequence of a stalling replisome or replication fork collapse on an encounter with a DNA lesion is the creation of ssDNA regions. This is a key event for the initiation of the SOS response. Accurate repair mechanisms, such as recombinational repair or excision repair, all of which involve or create ssDNA, try to cope with the damage before inducing mutagenic pol V. However, gaps persist²⁰ and disappear only very late during the SOS response. Coincidentally, pol V is also produced late in the SOS response, presumably as a last resort to facilitate cell survival.

We suggest that regulation of SOS mutagenesis requires elevated levels of ssDNA in *trans* for optimal pol V activation (Fig. 2). Simple access to a stalled replication fork and high amounts of RecA are insufficient for appreciable pol-V-catalysed synthesis. Mutagenesis is restricted to times of extensive DNA damage: for example, when numerous ssDNA gaps are present, thus increasing the chances for a ssDNA gap transactivator (Fig. 4b) to stimulate pol V on a separate replication fork. Any segment on ssDNA, if sufficiently long, can serve as a RecA filament platform (Fig. 4b). Once the damage is bypassed and extended, ssDNA disappears and pol V activities decline. The requirement for RecA during pol-V-catalysed TLS *in*

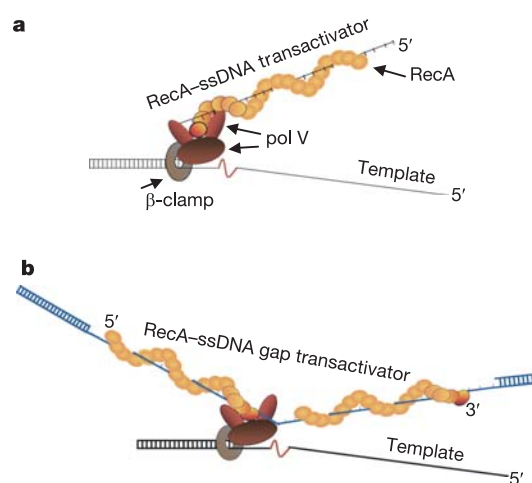


Figure 4 | Models depicting RecA-DNA transactivation of pol-V-catalysed translesion DNA synthesis. **a**, RecA-ssDNA (orange circles) is required for pol V (UmuD'₂C) to copy undamaged and damaged DNA templates. The essential feature of the transactivation model is that a RecA-DNA complex is formed on ssDNA that is not being copied by pol V. If RecA were to bind instead to the template strand, *cis* to pol V, then TLS would be blocked. **b**, A variation on the model shows how transactivation of pol V might occur by a RecA filament formed in gapped DNA (blue). A damaged DNA base is shown as a distortion in the template strand (brown squiggle).

vivo and *in vitro* seemed incompatible with the observation that RecA filaments formed in *cis* on the template being copied can block DNA synthesis^{12,16} (Supplementary Fig. 12). Activation of pol V by RecA–ssDNA in *trans* resolves this incompatibility and seems to constitute a new type of polymerase regulatory mechanism.

METHODS

Nucleotide incorporation on DNA primer templates. The polymerase activity was examined by extending primer/template hairpin DNA ³²P-labelled at the 5' end, or ³²P-labelled primed circular DNA, as indicated. Substrate DNA was preincubated with β -processivity-factor/ γ -clamp-loading complex (when present) and pol V before addition of RecA, *trans* DNA and dNTPs. Preincubation of *trans* DNA with RecA is redundant, as RecA will be attracted to DNA that is longer and/or in molar excess over primer/template DNA. RecA concentrations, *trans* DNA length or concentrations, and the presence of either ATP or ATP- γ S are indicated in the figures and figure legends. The sequences of template and transactivating DNAs are provided in Supplementary Fig. 13. For DNA beads, a biotinylated oligomer was attached to a streptavidin-coated magnetic bead (Bangs Laboratories) following the manufacturer's protocol. RecA was immobilized on the beads with ATP- γ S as described in Supplementary Fig. 10, and added to the reaction at a concentration of $\sim 1 \mu\text{M}$ in total DNA molecules. Unless indicated otherwise, reactions were carried out for 20 min at 37°C and the synthesis products were separated on a 20% denaturing polyacrylamide gel. The gel band intensities were measured by phosphorimaging with IMAGEQUANT software (Molecular Dynamics) and primer utilization was computed from the integrated gel band intensities of extended hairpin DNA or primer DNA. TLS was calculated by dividing the number of hairpins extended past the abasic site by the total number of extended hairpins. Transactivation kinetics curves (Supplementary Fig. 9) were analysed using Sigma Plot. For further details on the methods used in this study, see Supplementary Methods.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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LETTERS

Suppression of star formation in early-type galaxies by feedback from supermassive black holes

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Detailed high-resolution observations of the innermost regions of nearby galaxies have revealed the presence of supermassive black holes^{1–4}. These black holes may interact with their host galaxies by means of ‘feedback’ in the form of energy and material jets; this feedback affects the evolution of the host and gives rise to observed relations between the black hole and the host⁵. Here we report observations of the ultraviolet emissions of massive early-type galaxies. We derive an empirical relation for a critical black-hole mass (as a function of velocity dispersion) above which the outflows from these black holes suppress star formation in their hosts by heating and expelling all available cold gas. Supermassive black holes are negligible in mass compared to their hosts but nevertheless seem to play a critical role in the star formation history of galaxies.

The near-ultraviolet (NUV) detector of the Galaxy Evolution Explorer satellite GALEX⁶ covers a range in wavelength between 1,771 and 2,831 Å, and is extremely sensitive to young stellar populations. With it, we can detect small mass fractions (1–3%) of young stars formed within the last billion years^{7,8}. This high sensitivity allows us to trace ongoing residual star formation in present day early-type galaxies (ETGs). We select a volume-limited sample of visually inspected ETGs from the Sloan Digital Sky Survey⁹ that have been observed by GALEX. We remove active galactic nucleus (AGN) candidates from our sample to avoid confusion between the observed ultraviolet emissions from the AGN and from young stars¹⁰.

We use NUV–*r* colour as a probe of small amounts of recent star formation (RSF), and find that the fraction of ETGs that show signs of RSF is strongly correlated with the stellar velocity dispersion σ (hereafter ‘RSF– σ relation’), but not with the luminosity of the galaxy (see Fig. 1). The correlation with σ cannot be explained by a systematic trend of σ with internal extinction, because such a trend would be exactly opposite¹¹ to that observed.

We use a semi-analytic model of galaxy evolution in the Λ CDM paradigm to interpret these results^{12–14} (for model details see Supplementary Information). Our simulation includes all the systematic processes that affect galaxy evolution, and takes into account the different merger and star formation histories of galaxies¹⁵. When we run this simulation and investigate the predicted amount of recent star formation in low redshift ETGs, we find that all galaxies underwent enough star formation within the last billion years to be detected by the extremely sensitive GALEX NUV detector. Massive galaxies continued to gain cold gas as fuel for star formation from mergers with satellites and through the cooling of hot gas present in those galaxies.

This overproduction of stars in massive galaxies is a known problem in models of galaxy evolution. In order to prevent star formation, a shutdown mechanism is required: cold gas must be heated up and expelled to prevent it from turning into stars. Feedback from supernovae was once a prime suspect, but it turned out to be too low to be effective in massive galaxies that have deep potential wells¹⁶. All the systematics implicit in the Λ CDM paradigm, including feedback from supernovae, have already been included in our simulation, so we turn to feedback from AGN powered by supermassive

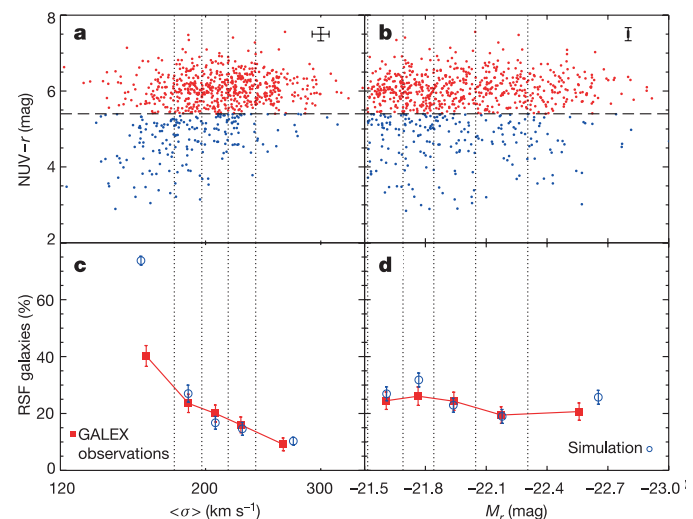


Figure 1 | The relationship between stellar velocity dispersion, luminosity and the fraction of star-forming early-type galaxies. **a, b**, The observed ultraviolet colour– σ (**a**) and colour– M_r (**b**) relations. NUV, apparent magnitude in the near ultraviolet; r , apparent magnitude in the band between 5,500 and 7,000 Å; σ , stellar velocity dispersion; M_r , absolute magnitude. The observational errors show 1σ . In **a**, the fraction of blue early-type galaxies declines sharply as a function of σ . **c, d**, The derived fractions of RSF (recent star formation) galaxies as a function of σ (**c**) and M_r (**d**). In red, we show the observed fractions, which include those early-type galaxies so faint in the ultraviolet that they are not detected by the GALEX NUV detector. The blue points are the fractions of those galaxies in the model that correspond to having had between 1 and 3% of stellar mass growth within the last 1 billion years. Error bars show Poisson errors. The vertical dotted lines delineate the equal-number σ bins.

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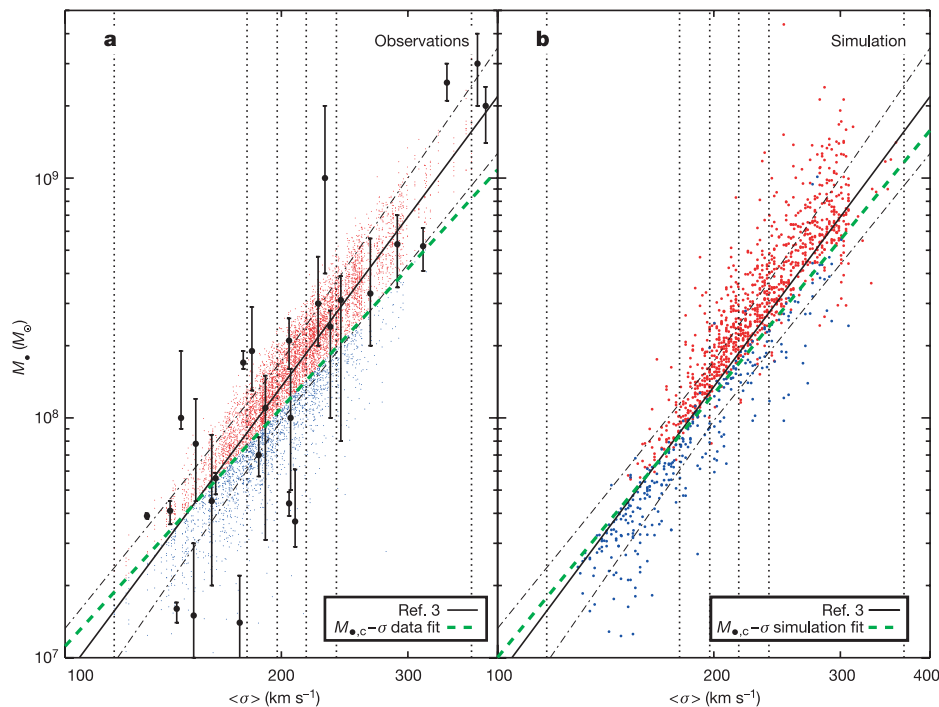


Figure 2 | The black-hole mass- σ and the critical supermassive black-hole mass- σ relation. **a**, The observed black-hole mass $M_{\bullet}$ - σ data points (black filled circles) with their errors taken from ref. 3. The solid line is the best fit to the $M_{\bullet}$ - σ relation, with the dash-dotted line showing the 1σ scatter. Based on this relation, we perform a Monte-Carlo simulation for our galaxies (small dots). We colour these simulated galaxies depending on whether they lie above or below the critical relation (dashed green line) in red (quiescent)

and blue (RSF) that we derived by fitting the RSF fraction to the observed values (Fig. 1). The vertical lines delineate the equal-number σ bins of the data. **b**, We show our semi-analytic galaxy models adopting $M_{\bullet,c} \propto \sigma^{3.65}$ (dashed green line), in comparison to the empirical $M_{\bullet}$ - σ relation. Red and blue dots are the model galaxies that respectively did and did not have recent star formation in the last billion years.

black holes as the remaining most likely mechanism sufficiently powerful to shut down star formation in massive galaxies to reproduce the results of our observations^{17–19}.

Energetic outflows caused by gas falling onto the supermassive black holes thought to reside at the centres of galaxies can self-regulate the growth of the black hole and the star formation in the galaxy by heating the available gas and blowing it out⁵. Numerical simulations have shown that the energy input from a black hole in such an AGN phase is capable of doing this²⁰. As supernova feedback is incapable of producing the quiescent galaxies we observe with GALEX, we conclude that AGN feedback is the most likely process by which the available gas is heated and expelled in massive ETGs.

In addition to this, the nature of the observed correlation with σ (Fig. 1) allows us to infer more about the way in which AGN feedback regulates galaxy evolution. The masses of black holes correlate strongly with various properties of the host galaxies, and the relation thought to have the smallest intrinsic scatter is that between black-hole mass ($M_{\bullet}$) and the velocity dispersion σ as a proxy for the depth of the gravitational potential^{1,3,4}. By considering the maximum strength of an AGN given in terms of the Eddington luminosity $L_{\text{Edd}} \propto M_{\bullet}$ and expressing the gravitational potential depth by the stellar velocity dispersion σ , it is possible to derive a minimum black-hole mass for which the outflow is capable of depleting all gas from the potential⁵. According to our observations, massive ETGs that have large values of σ are on average less likely to have undergone RSF compared to less massive ETGs: the ‘RSF- σ relation’. Thus, for a given velocity dispersion, we assume that there is a critical supermassive black hole mass, $M_{\bullet,c}$, above which the feedback powered by the black hole is sufficiently powerful to heat up and/or expel all gas in the host galaxy, thus preventing it from forming any stars, even at the 1% level.

The observed intrinsic scatter in the $M_{\bullet}$ - σ relation gives a range of black-hole masses for a given value of σ . Hence, in order to reproduce the decreasing fraction of RSF ETGs with increasing σ , the $M_{\bullet,c}$ - σ relation should be less steep than the $M_{\bullet}$ - σ relation itself. We can derive an empirical $M_{\bullet,c}$ - σ relation from our observed trend assuming a slope and scatter for the $M_{\bullet}$ - σ relation. The latter relation is usually expressed as a power law of the form $M_{\bullet} = a_0(\sigma/200)^{a_1}$, so we also try to find such parameters a_0 and a_1 in the new $M_{\bullet,c}$ - σ relation that result in the observed ‘RSF- σ relation’. We generate black-hole masses for the galaxies in our sample by putting their velocity dispersions into the $M_{\bullet}$ - σ relation given in ref. 3 and perturbing the black-hole masses by the scatter of the relation; we do this ten times for each galaxy (Fig. 2a). Then we vary a_0 and a_1 until we find the best match to the observed RSF- σ relation. We find that with 95% confidence $M_{\bullet,c} = (1.05^{+0.07}_{-0.12}) \times 10^8 (\frac{\sigma}{200})^{3.2^{+0.5}_{-0.6}}$. As we are studying a volume and magnitude-limited sample, the $M_{\bullet,c}$ - σ relation derived from the Monte Carlo simulation is only an approximation; we need to perform full simulations, which are complete in σ as well as in absolute magnitude, to derive the actual $M_{\bullet,c}$ - σ relation.

We perform a more realistic simulation using our semi-analytic models of galaxy evolution in order to test the plausibility of the hypothesis of a critical black-hole mass controlling star formation via AGN feedback. This is a standard semi-analytic simulation in the Λ CDM paradigm that already includes various heating and cooling processes (for example, supernovae). Our model does indeed reproduce the observed trend, but only when AGN feedback is included, and the best agreement with the observations (Fig. 1) is achieved by the relation $M_{\bullet,c} = 1.26 \times 10^8 (\frac{\sigma}{200})^{3.65}$ (Fig. 2b), which is in good agreement with our initial guess. The agreement in the lowest σ -bin is poorer than in the others owing to an increased scatter in our simulated Faber–Jackson relation relative to the observed one²¹.

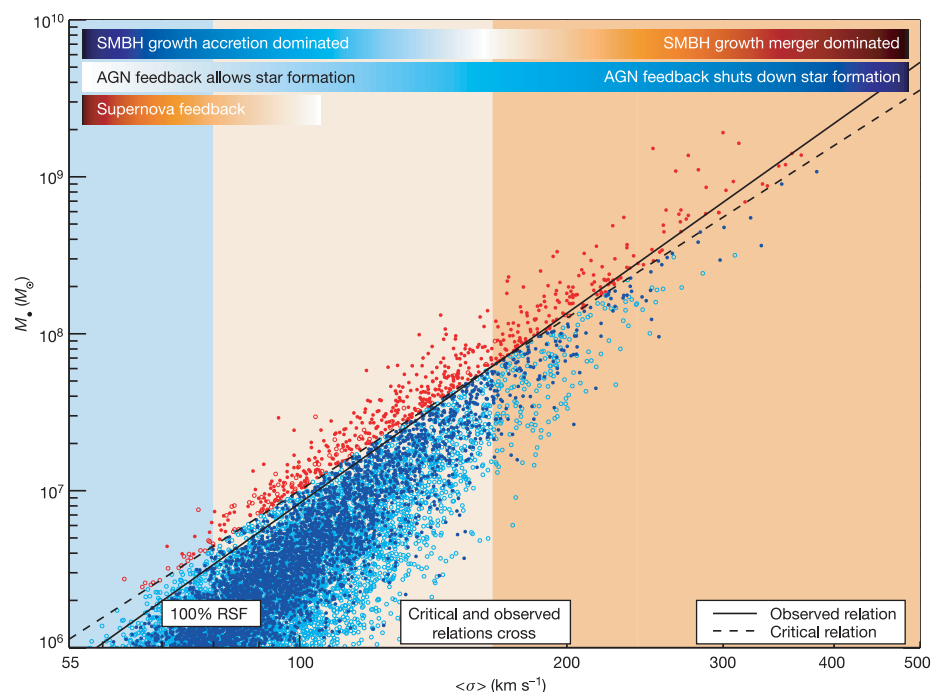


Figure 3 | A schematic view of how the critical supermassive black hole mass- σ relation regulates galaxy evolution. The black-hole masses and velocity dispersions of our simulated galaxies down to $\sigma \approx 50 \text{ km s}^{-1}$. The filled circles are early-type galaxies, while open circles are late-type galaxies. Red and blue symbols denote quiescent and RSF galaxies, respectively. We

show the observed $M_{\bullet}-\sigma$ relation (solid line), together with the best semi-analytic model critical relation (dashed). We indicate the various regimes by colour; see text for details. SMBH, supermassive black hole; AGN, active galactic nuclei.

The supermassive black holes grown in our simulation have an intrinsic scatter of a factor of 1.5 in mass and are in good agreement with the relation suggested in ref. 3.

As our $M_{\bullet,c}-\sigma$ relation is less steep than the $M_{\bullet}-\sigma$ relation, we expect an increasing fraction of star-forming ETGs with decreasing velocity dispersion until eventually no AGN will be sufficiently powerful to shut down all star formation. The NUV- V (optical) and NUV- H (near-infrared) colour-magnitude relations for the early-type population of the Virgo cluster shows precisely this kind of trend: low luminosity—and therefore less massive—Virgo ETGs are increasingly bluer and eventually all lie below the RSF cut-off²². We use stellar models²³ to convert our RSF criterion to the NUV- V colours of the Virgo galaxies, and find that below $\sigma \approx 80 \text{ km s}^{-1}$ all early-types are classified as RSF. Our simulations agree with this cut-off very well.

The possible implications of the $M_{\bullet,c}-\sigma$ relation for galaxy formation and the growth of black holes can be summarized by three main regions (Fig. 3). The first is the low- σ region, in which the majority of black holes are too small to be able to halt star formation in their host galaxies by feedback during their initial accretion phase. During this phase, black holes grow mainly by accreting gas at the Eddington rate and are only limited in their growth by the amount of available fuel. Star formation, on the other hand, is regulated by the energy output from supernovae into the interstellar medium and will be proportional to the surface density of the gas²⁴. This region extends up to $\sigma \approx 80 \text{ km s}^{-1}$, as suggested earlier. The second region is the intermediate- σ region, at $80 \text{ km s}^{-1} < \sigma < 240 \text{ km s}^{-1}$. Black holes are now becoming on average massive enough to be able to terminate star formation by their feedback and at the same time limit their available fuel. The mass growth of the black holes is still dominated by accretion, but mergers between black holes start playing an increasingly important role. Most host galaxies will be identified as AGN or quasars when observed during the accretion phase onto the black hole. We subdivide this region into two: at the lower end, a minority of galaxies will have critical black holes; the

black holes still grow mostly via accretion. At the high end, most black holes are critical and so most of the mass growth now occurs via mergers. The boundary between these two is at the intersection between the $M_{\bullet}-\sigma$ and the critical relation, that is, at $\sigma \approx 165 \text{ km s}^{-1}$. The last region is the high- σ region. For galaxies with $\sigma > 240 \text{ km s}^{-1}$, the vast majority of black holes are massive enough to halt all star formation and so star formation becomes virtually prohibited in such massive galaxies. Both the galaxy and the supermassive black holes now mainly grow via dry mergers²⁵. Further observations of nearby galaxies hosting supermassive black holes in combination with star formation estimates will help to shed more light into the scenario we propose, and will place a strong test for the existence of 'a critical black-hole mass' in galaxies.

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LETTERS

Transient pulsed radio emission from a magnetar

Fernando Camilo¹, Scott M. Ransom², Jules P. Halpern¹, John Reynolds³, David J. Helfand¹, Neil Zimmerman¹ & John Sarkissian³

Anomalous X-ray pulsars (AXPs) are slowly rotating neutron stars with very bright and highly variable X-ray emission that are believed to be powered by ultra-strong magnetic fields of $>10^{14}$ G, according to the 'magnetar' model¹. The radio pulsations that have been observed from more than 1,700 neutron stars with weaker magnetic fields have never been detected from any of the dozen known magnetars. The X-ray pulsar XTE J1810–197 was revealed (in 2003) as the first AXP with transient emission when its luminosity increased 100-fold from the quiescent level²; a coincident radio source of unknown origin was detected one year later³. Here we show that XTE J1810–197 emits bright, narrow, highly linearly polarized radio pulses, observed at every rotation, thereby establishing that magnetars can be radio pulsars. There is no evidence of radio emission before the 2003 X-ray outburst (unlike ordinary pulsars, which emit radio pulses all the time), and the flux varies from day to day. The flux at all radio frequencies is approximately equal—and at >20 GHz XTE J1810–197 is currently the brightest neutron star known. These observations link magnetars to ordinary radio pulsars, rule out alternative accretion models for AXPs, and provide a new window into the coronae of magnetars.

We observed the position of XTE J1810–197 (ref. 4) on 17 March 2006 for 4.2 hours with the Parkes telescope at a central frequency $\nu = 1.4$ GHz, using parameters identical to those used in the Parkes multibeam pulsar survey⁵. Pulsations with period $P = 5.54$ s were easily detected, with period-averaged flux density $S_{1.4} = 6$ mJy and a narrow average profile with full-width at half-maximum of 0.15 s (Fig. 1). We detected individual pulses from virtually every rotation of the neutron star (see Fig. 2). These are composed of ≤ 10 -ms-wide sub-pulses with peak flux densities up to ≥ 10 Jy and follow a differential flux distribution approximated by $\text{dlog}N = -\text{dlog}S$, with no giant pulses like those observed from the Crab pulsar⁶.

A timing model accounting for every turn of the neutron star during the period 17 March–7 May yields barycentric $P = 5.54024870$ s $\pm$ 20 ns on MJD 53855.0 and $\dot{P} = (1.016 \pm 0.001) \times 10^{-11}$, with root-mean-square residual $\sigma = 5$ ms. We use this to set constraints on any putative companion to the AXP by requiring the light-travel-time across the projected orbital semi-major axis to be less than σ . From Kepler's third law, with assumed neutron star mass $1.4M_{\odot}$, the upper limits on the minimum companion mass lie in the range ~ 0.003 – $0.03M_{\odot}$ for orbital periods in the range 2 h–5 min, effectively ruling out the existence of any Roche lobe-filling star orbiting this AXP. The delay in pulse arrival times measured between 2.9 and 0.7 GHz implies an integrated column density of free electrons between the Earth and XTE J1810–197 of $178 \pm 5 \text{ cm}^{-3} \text{ pc}$. Together with a model for the Galactic distribution of free electrons⁷, the distance to XTE J1810–197 is $D \approx 3.3$ kpc (here we use $\approx$ to indicate a quantity known to within about a factor of two or better), consistent with X-ray- and optically-derived estimates of 2.5–5 kpc (refs 8–10).

In the original detection of radio emission from XTE J1810–197

with the Very Large Array (VLA) in January 2004, $S_{1.4} = 4.5$ mJy (ref. 3). In February 2006 at the VLA we measured $S_{1.4} = 12.9$ mJy, a considerable increase in flux over a period of two years. However, further observations (see Table 1) reveal large fluctuations: at Parkes, $S_{1.4}$ can vary by factors of about two from one day to the next, although no significant variations are detected within observing sessions lasting up to four hours. These flux variations are completely inconsistent with expectations from interstellar scintillation^{11,12} and are thus, remarkably for a pulsar, intrinsic to XTE J1810–197. At the VLA, $S_{8.4}$ shows variations by similar factors within 20 min. This more rapid variation at high frequencies is confirmed in pulsed observations with the Green Bank Telescope (GBT). However, at $\nu \geq 9$ GHz diffractive interstellar scintillation^{11,12} plays a significant role⁷. At present we cannot rule out a non-pulsed component. The maximum size of any extended emission is $5 \times 10^{15} (D/3 \text{ kpc}) \text{ cm}$, based on the 0.25-arcsec resolution of our VLA image at 8.4 GHz, from which we measure a position for XTE J1810–197 of right ascension = 18 h 09 min 51.087 s $\pm$ 0.001 s and declination = $-19^{\circ} 43' 51.93'' \pm 0.02''$, in the J2000.0 equinox.

The radio spectrum of XTE J1810–197 is quite flat over a factor of 60 in frequency. We have made independent simultaneous measurements of flux at multiple frequencies (see Table 1), all of which are consistent with spectral index $\alpha \approx -0.5$, where $S_{\nu} \propto \nu^{\alpha}$. Ordinary pulsars have mean $\alpha = -1.6$, and fewer than 10% have $\alpha > -0.5$ (ref. 13). With an unremarkable average flux at $\nu \sim 1$ GHz (here we use $\sim$ to indicate a quantity known to within about an order of magnitude), by $\nu \geq 20$ GHz XTE J1810–197 is brighter than every other known neutron star. Its assumed isotropic radio luminosity up to 42 GHz is about $2 \times 10^{30} \text{ erg s}^{-1}$, compared to the spin-down luminosity of about $2.4 \times 10^{33} \text{ erg s}^{-1}$.

Radiation from XTE J1810–197 is highly polarized: $89 \pm 5\%$ of the total-intensity 8.4-GHz flux measured at the VLA is linearly polarized. Also, a 1.4-GHz Parkes observation shows linear polarization that tracks the total-intensity pulse profile at a level of 65%.

XTE J1810–197 is located within the boundaries of the Parkes multibeam pulsar survey area⁵. We have analysed archival raw survey data from the two telescope pointings nearest to the pulsar position. No pulsar signal was detected in average-pulse analyses, corresponding to $S_{1.4} \leq 0.2$ mJy from these observations made in 1997 and 1998 (see Table 1). This limit is $<10\%$ of the flux from the pulsar since 2004, suggesting that the extraordinary radio emission from XTE J1810–197 turned on as a result of the events accompanying the X-ray outburst observed in early 2003 (ref. 2).

However, about 10% of ordinary pulsars have specific radio luminosity $L_{1.4} \equiv S_{1.4} D^2 \leq 2 \text{ mJy kpc}^2$, the approximate limit for XTE J1810–197 before the outburst. For example, the pulsar PSR J1718–3718, with $L_{1.4} \approx 4 \text{ mJy kpc}^2$ and $P = 3.3$ s, has inferred surface dipole magnetic field strength $B = 7 \times 10^{13}$ G, higher than that of one magnetar (for XTE J1810–197, $B \approx 2.4 \times 10^{14}$ G). This source has X-ray properties apparently similar to those of XTE J1810–197 in

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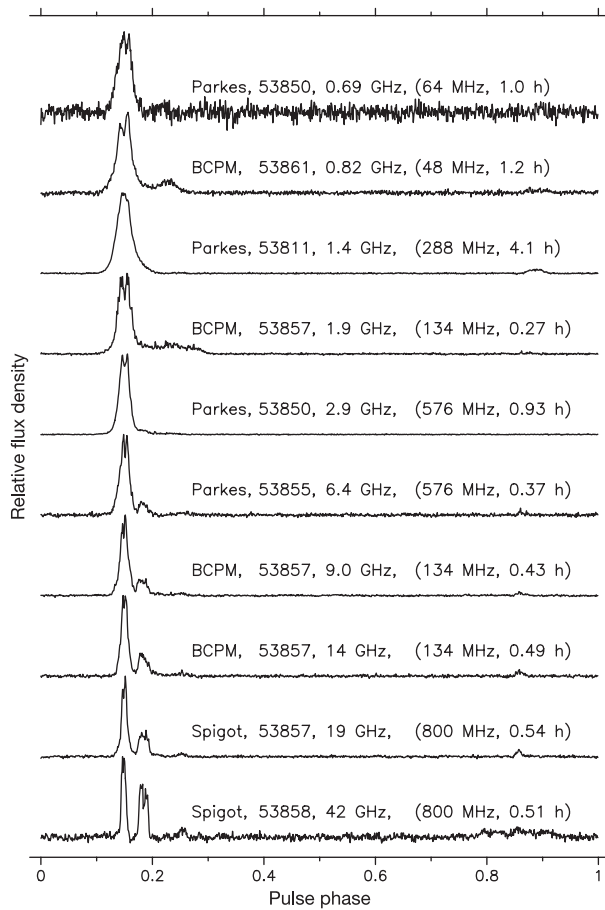


Figure 1 | Average radio pulse profiles of XTE J1810-197 at frequencies 0.7–42 GHz. Full-period (5.54 s) profiles are displayed with 1,024-bin resolution in order of ascending frequency from top to bottom, and aligned by fitting the main pulse peak with a gaussian. Above each trace we list the equipment, date (MJD), central frequency, nominal bandwidth, and integration time used to obtain each profile. In some cases a small number of frequency channels corrupted by radio-frequency interference have been omitted in constructing the profiles. The Parkes 1.4-GHz (discovery) observation shows a small pulse preceding the main pulse by about 0.25 in phase. This precursor pulse is not detected on some days. These and other changes may be related to the well-known emission of separate “modes”²⁷ displayed by some ordinary pulsars, or could be more exotic, for instance due to magnetic field reconfiguration or magnetospheric currents varying on timescales of days to weeks. The separation between the two peaks of the main pulse component appears to decrease with increasing frequency, possibly akin to what is observed in some ordinary pulsars, for which the separation is interpreted as reflecting a decreasing emission altitude with increasing frequency. For observations at Parkes we used an analogue filterbank with one-bit digitization and a high-pass filter of time constant ≈ 0.9 s that significantly distorts the measured profiles. We corrected for this using the prescription given in ref. 5. At GBT we used both the BCPM²⁸ and Spigot²⁹, and no such coarse artefacts resulted.

quiescence¹⁴. This raises the prospect that XTE J1810-197 could have generated familiar radio pulsar emission before 2003, marking a plausible direct link between ordinary pulsars and magnetars. In addition, a recently discovered class of radio-bursting neutron stars¹⁵ includes at least one object whose field strength is comparable to that of some magnetars; it too has X-ray properties resembling those of XTE J1810-197 in quiescence¹⁶. We therefore also searched the archival radio data for bright individual pulses, but found none.

Observable magnetospheric radio emission requires a coherent mechanism, inferred to be curvature radiation whose characteristic

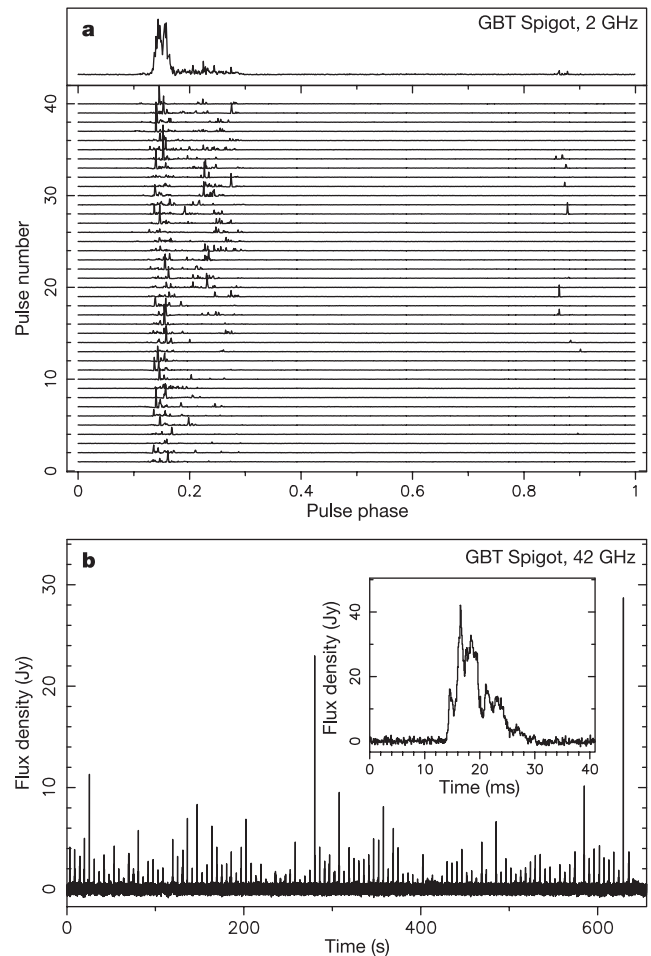


Figure 2 | Single pulses from XTE J1810-197 at frequencies of 2 and 42 GHz. We detect single pulses from most rotations of the neutron star irrespective of frequency. **a**, We show here a typical set of 40 consecutive single pulses from our GBT observation at 2 GHz on MJD 53857, where each row represents the full pulse phase displayed with 5.4-ms resolution (1,024 bins). The sum of all 40 pulses is displayed at the top. Sub-pulses (for which we have found no evidence of “drifts”³⁰) with typical width ≤ 10 ms arrive at different phases and gradually build up the average profile—which, however, appears different in observations 8 days later, with the shoulder at phase 0.2–0.3 essentially missing. It is unclear whether this behaviour is similar in detail to what is observed in ordinary pulsars. **b**, A train of about 115 consecutive single pulses detected at a frequency of 42 GHz with the GBT, displayed with 1.3-ms resolution. For display purposes, we have removed large-amplitude power variations with timescales of ≥ 10 s, probably of atmospheric origin, by high-pass-filtering the data. The flux-density scale is uncertain by a factor of about 2. Inset, a 40-ms-long detail of the brightest pulse from the main panel, displayed with full 81.92-μs Spigot resolution. The pulse has structure with features as narrow as ≈ 0.2 ms.

frequency $3\gamma^3 c/4\pi r$ falls in the gigahertz range for secondary particle Lorentz factors $\gamma \sim 10^3$ and radii of curvature r of the order of the light-cylinder radius $r_{lc} = cP/2\pi$ or smaller. In ordinary pulsars, the required electron–positron pairs can be produced only on the open field-line bundle at high altitude above the surface where electric potentials of $\sim 10^{12}$ V accelerate particles that emit gamma-rays with energies exceeding the pair-creation threshold. We cannot exclude that the handful of known magnetars employ this mechanism, because their long periods imply small active polar caps and narrow beams that may miss the observer for random orientations. Additionally, magnetars have mechanisms and locations for pair creation that are not available to ordinary pulsars^{17–19}. Magnetar activity is generated by the sudden or continual twisting of the

Table 1 | Variable radio flux densities of XTE J1810–197

Instrument	Frequency, ν (GHz)	Date (MJD)	Flux density, S_ν (mJy)
Parkes	1.4	50747	≤ 0.25
Parkes	1.4	51152	≤ 0.15
VLA	1.4	53019	4.5 ± 0.5
VLA	1.4	53794	12.9 ± 0.2
Parkes	1.4	53811	6.0
Parkes	1.4	53850.9 ^a	6.8
Parkes	1.4	53851.9	13.6
Parkes	1.4	53852.9	13.2
Parkes	1.4	53855.9 ^b	8.7
GBT (BCPM)	1.4	53862.4	5.1
Parkes	1.4	53862.9	7.8
VLA	1.4	53872	3.3 ± 0.2
VLA	1.4	53874	6.6 ± 0.5
VLA	1.4	53875	4.5 ± 0.5
VLA	1.4	53877	8.6 ± 0.7
VLA	1.4	53879	5.8 ± 0.6
GBT (BCPM)	1.9	53857.3 ^c	6.3
GBT (BCPM)	1.9	53865	6.0
Parkes	0.69	53850.9 ^a	7.4
GBT (BCPM)	0.82	53861.2	5.5
Parkes	2.9	53850.9 ^a	7.4
Parkes	6.4	53855.9 ^b	3.9
VLA	8.4	53828	9.0 ± 0.1
GBT (BCPM)	9.0	53857.4 ^c	3.0
GBT (BCPM)	14	53857.4 ^c	2.1
GBT (Spigot)	19	53857.5 ^c	1.9
GBT (Spigot)	42	53858.4	5.9

We list the flux densities from all observations of XTE J1810–197 with Parkes and GBT, as well as detections with the VLA (see also ref. 3). The X-ray outburst from XTE J1810–197 occurred between MJDs 52595 and 52662 (ref. 2). We searched for pulsations from the two pointings nearest to the neutron star in the Parkes multibeam pulsar survey³ (offset by 8 and 5 arcmin, respectively, where the telescope beam has full-width at half-maximum of 14.5 arcmin). A null result provides the given upper limits. Multiple observations at 1.4 and 1.9 GHz are listed by frequency chronologically in order to aid characterization of flux variations. Following this, we list observations in order of ascending frequency. VLA data are flux-calibrated with high precision using the flux density standard 3C286. Parkes and GBT fluxes are measured using the radiometer equation by comparing the area under each average pulse profile to the root-mean-square baseline and adopting known observing system characteristics. Absolute uncertainties for these fluxes are typically about 30% for $\nu \leq 9$ GHz, but increase for $\nu \geq 14$ GHz (we have applied corrections to account for the opacity of the atmosphere). Owing to poor atmospheric conditions, S_{42} is known to within a factor of only about 2. Relative changes in $S_{1.4}$ can be gauged with approximately 10% uncertainty, and clear variations (by factors of ≈ 2 on timescales of ~ 1 day) are observed at Parkes and also at VLA. On timescales typically up to one hour there is no evidence for intra-day variations in flux from any observation at $\nu \leq 9$ GHz. Conversely, all observations above this frequency show evidence for variability on timescales of several minutes. For example, on MJD 53828, $S_{8.4}$ was measured in successive 13-min scans across a 100-MHz band to be 7.4, 11.2, 9.6, 10.5, 5.1 and 8.3 mJy (all uncertainties ≤ 0.2 mJy). At these high frequencies, diffractive interstellar scintillation^{11,12} is at least partly responsible for the observed flux variations. We denote by superscripts a, b and c the three days on which we obtained multi-frequency data, from which the spectral index can be estimated with some accuracy: $\alpha \approx -0.5$.

external field lines into a non-potential configuration that maintains, by induction, strong long-lived currents flowing along them²⁰. With required charge densities greatly exceeding the co-rotation value²¹ for a dipole field, an electric potential of $\sim 10^9$ V is established and self-regulated by pair creation. In these conditions, electrons and positrons are accelerated to $\gamma \sim 10^3$, and pairs can be created either via resonant cyclotron scattering of thermal X-rays in the strong magnetic field or thermally in the heated atmosphere at the footpoints of the twisted field lines. This affords the possibility that magnetar radio emission is generated on closed field lines and beamed into a wide range of angles. Because most of the energy of the twisted field is contained within a few stellar radii, the frequencies of coherent emission from magnetars could be greater than those of ordinary pulsars, possibly accounting for the flat radio spectrum of XTE J1810–197. Also, it has been proposed that the observed optical and infrared emission from magnetars is coherent emission from plasma instabilities above the plasma frequency^{22,23}, and even that radio or submillimetre emission could be so generated²⁴. The extrapolated radio spectrum of XTE J1810–197 exceeds its observed infrared fluxes²⁵, so that the radio and infrared emission may or may not have the same origin.

Radio emission as currently observed from XTE J1810–197 was evidently not present during the historical quiescent X-ray state that persisted for at least 24 years before the outburst²⁶. Following this, the onset of radio emission could have been delayed until the plasma density declined to a value much less than is generally found in persistent magnetars. The hard and soft X-ray fluxes are now decaying exponentially with timescales of ≈ 300 and ≈ 900 days, respectively⁸, while strong radio emission is still observed more than three years after the X-ray turn-on. We infer that radio emission will cease after the transient X-ray components (and implied magnetospheric currents) have subsided, but it is difficult to know whether this transition is imminent, or will take many years.

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LETTERS

Nuclear isomers in superheavy elements as stepping stones towards the island of stability

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A long-standing prediction of nuclear models is the emergence of a region of long-lived, or even stable, superheavy elements beyond the actinides. These nuclei owe their enhanced stability to closed shells in the structure of both protons and neutrons^{1–3}. However, theoretical approaches to date do not yield consistent predictions of the precise limits of the ‘island of stability’; experimental studies are therefore crucial. The bulk of experimental effort so far has been focused on the direct creation of superheavy elements in heavy ion fusion reactions, leading to the production of elements up to proton number $Z = 118$ (refs 4, 5). Recently, it has become possible to make detailed spectroscopic studies^{6,7} of nuclei beyond fermium ($Z = 100$), with the aim of understanding the underlying single-particle structure of superheavy elements. Here we report such a study of the nobelium isotope ²⁵⁴No, with 102 protons and 152 neutrons—the heaviest nucleus studied in this manner to date. We find three excited structures, two of which are isomeric (metastable). One of these structures is firmly assigned to a two-proton excitation. These states are highly significant as their location is sensitive to single-particle levels above the gap in shell energies predicted at $Z = 114$, and thus provide a microscopic benchmark for nuclear models of the superheavy elements.

One key gap in our understanding of the chemical building blocks of the Universe is the question of the heaviest element that can exist. Intimately linked to this question is the problem in nuclear physics of the shell stabilization of superheavy nuclei. In a simplistic model of the nucleus, the repulsive force between more than 100 positively charged protons is sufficient to overcome the attractive strong nuclear force and induce the nucleus to fission. However, protons and neutrons fall into shell structures which, in analogy to the enhanced stability of noble gases, give extra binding to nuclei in a closed shell configuration. The largest binding comes from a coincidence of closed shells for both protons and neutrons, as in ¹⁶O ($Z = N = 8$), ⁴⁰Ca ($Z = N = 20$), ¹³²Sn ($Z = 50, N = 82$) and ²⁰⁸Pb ($Z = 82, N = 126$); here N is the neutron number. The successful explanation of these nuclear magic numbers ($Z = N = 2, 8, 20, 28, 50, 82$ and $N = 126$) via a large spin–orbit splitting led to the 1963 Nobel prize in physics for Goeppert-Mayer and Jensen. In superheavy nuclei the spin–orbit interaction can open substantial shell gaps—for example, between the proton $2f_{7/2}$ – $2f_{5/2}$ spin–orbit partners. A nucleus with 114 protons will fill all orbitals up to the $2f_{7/2}$ shell, and it is the strength of the spin–orbit interaction that determines the size of the $Z = 114$ gap, as schematically shown in Fig. 1.

Modern theoretical approaches disagree on the size and position of this shell gap. The microscopic-macroscopic models with various parameterizations of the nuclear potential predict $Z = 114$ and $N = 184$. Calculations using self-consistent mean-field approaches have been performed by several authors and broadly fall into two categories, namely, relativistic and non-relativistic approaches. In both cases, the splitting between the $2f_{7/2}$ – $2f_{5/2}$ spin–orbit partners is not sufficient to open a gap. Most non-relativistic mean-field calculations favour $Z = 124, 126$ and $N = 184$, while the relativistic mean-field models show that the effects of magic numbers of single nucleonic configurations valid in lower mass magic nuclei (Sn, Pb,

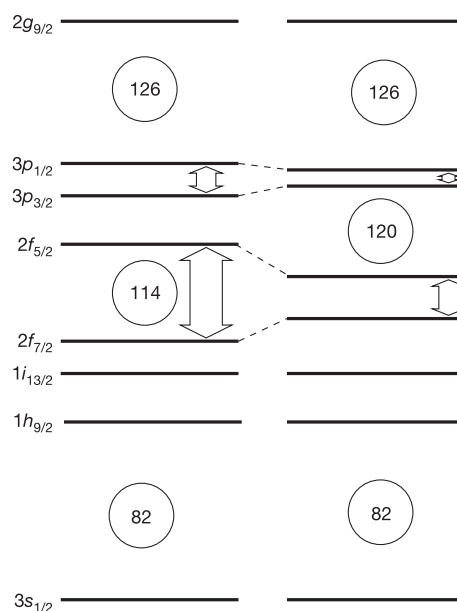


Figure 1 | Schematic illustration of the spherical single-proton orbitals in the region of superheavy elements. The strength of the spin–orbit interaction determines the size of the gaps at 114 and 120. Left, large spin–orbit coupling; right, reduced spin–orbit coupling. The spherical levels are labelled with the radial quantum number, n , and the orbital and total angular momentum, l and j respectively, in standard spectroscopic form, nl_j . The circled numbers indicate the resulting energy gaps. Dashed lines connect corresponding levels.

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and so on) are dissolved in favour of more extended regions of additional shell stabilization¹, centred mainly around $Z = 120$, $N = 172$, 184 or $Z = 126$, $N = 184$. Theoretical and experimental progress in this area has recently been reviewed by a number of authors^{1–8}.

Experimental data for superheavy elements are scarce because the production cross-sections for these elements drop rapidly with increasing proton number, down to the 1 picobarn (1 pb) level for element 112 (ref. 9). With this cross-section, a few atoms per month can be produced. This means that the majority of data available yield integral quantities such as half-lives, decay modes and α -decay Q values, but are insensitive to the details of the nucleonic shell structure. However, recently it has become possible to perform spectroscopic studies in nuclei approaching the island of stability, such as ^{254}No ($Z = 102$, $N = 152$). Nuclei in this region are deformed, and the degenerate spherical single-particle orbitals split in a well defined and adequately understood manner into components according to the projection of the angular momentum onto the symmetry axis of the nucleus, the K quantum number. Orbitals originating above the relevant spherical proton shells (such as $2f_{5/2}$) come close to the Fermi level in a deformed nucleus and thus play a key role in the formation of excited states.

In nuclei with even numbers of protons and neutrons, the ground state always has $K = 0$. Configurations with large values of K thus require a decay path to the ground state that changes this projection gradually. If such intermediate configurations do not exist, the high- K state becomes isomeric¹⁰. This gives a very clear and unique experimental signature because the isomeric states can readily be identified from their decay times and paths. It has been suggested that in superheavy nuclei the occurrence of isomeric states may be a key factor in the enhanced stability of these nuclei, with isomeric lifetimes exceeding those of the ground states^{1,11}.

In this work we have chosen the $Z = 102$ nucleus ^{254}No as it provides an ideal laboratory for these studies. It is produced with a reasonable cross-section of $2\ \mu\text{b}$, which allows a production rate of 200 atoms per hour, sufficient for detailed spectroscopic studies. Previous measurements¹² in the 1970s tentatively proposed an isomeric configuration in ^{254}No whose existence was recently corroborated through the observation of conversion electron cascades¹³. ^{254}No also has attracted considerable theoretical interest^{14–17} as it became the first transfermium nucleus whose rotational structure was established in 1999¹⁸.

In view of the profound implications of this isomer for nuclear models, it is important not only to verify its existence and determine the half-life, but also to elucidate the decay path and determine its configuration. We report here the findings of experiments performed at the Accelerator Laboratory of the University of Jyväskylä, Finland. Similar, but not identical, results on the isomers in ^{254}No have been obtained from experiments at Argonne National Laboratory¹⁹.

The ^{254}No ions were produced via the $^{208}\text{Pb}(^{48}\text{Ca}, 2n)^{254}\text{No}$ reaction at a beam energy of 219 MeV. They were separated from the beam and unwanted reaction products in the gas-filled recoil separator RITU²⁰ before being implanted in a double-sided position sensitive Si detector (DSSD) at the heart of the GREAT spectrometer²¹. Long lived isomeric nuclei then decayed to the ground state, emitting γ -rays, X-rays, and conversion and Auger electrons. Conversion and Auger electrons were detected in the same pixel of the DSSD where they summed to a total deposited energy of up to 600 keV. This provided a clean signal for the decay of the isomer, a method originally suggested by Jones²². The γ -rays and X-rays were detected in prompt coincidence with this electron signal in a large segmented planar Ge detector in close proximity to the DSSD and a large Clover Ge detector outside the detector chamber. Finally, the ground state of ^{254}No decays via its characteristic 8.1 MeV α -decay with a half-life of $T_{1/2} = 51.2 \pm 0.4\ \text{s}$, and is recorded in the same DSSD pixel. It is this characteristic sequence of implanted recoil, one or more isomeric decays followed by the characteristic α -decay all in the same detector

pixel that allows us to firmly assign the observed isomeric decays to ^{254}No .

Figure 2 shows the time distribution of the electron signal following the recoil implantation. The long-lived isomer with $T_{1/2} = 266 \pm 2\ \text{ms}$ is clearly seen (Fig. 2a inset). In addition a new, short-lived isomer with $T_{1/2} = 184 \pm 3\ \mu\text{s}$ is observed (Fig. 2b inset) to feed the 266 ms isomer. From the electron energy distributions (Fig. 2a, b) and the γ -ray spectra (Fig. 2c, d) associated with these isomers, one can deduce the decay path in some detail; we propose the level scheme shown in Fig. 3. The 266 ms isomer decays via a 53 keV electric dipole (E1) transition into an excited two-quasi-particle band feeding the 7^+ rotational band member. The E1 character of this transition is deduced from the observed very small internal conversion coefficient. From the γ -ray intensity (I_γ) ratios between stretched electric quadrupole (E2) and mixed E2/M1 transitions, $R_1 = I_\gamma(81\ \text{keV})/I_\gamma(150\ \text{keV})$ and $R_2 = I_\gamma(69\ \text{keV})/I_\gamma(126\ \text{keV})$, a value for the g -factor of the band can be extracted assuming the validity of the rotational model. This g -factor is sensitive to the nucleonic configuration. We find $g_K = 0.87 \pm 0.14$. This band in turn decays via low energy magnetic dipole (M1) and E2 transitions to the band head before decaying to the ground state band via two prominent transitions at 841 and 943 keV.

The band-head spin was earlier determined to be $J = 3$ (ref. 23). Here the multipolarity of the 841 and 943 keV γ -ray transitions has been determined to be M1, which firmly establishes the band-head spin and parity as $J^\pi = 3^+$. As the full decay path is known, the isomer can be placed at an excitation energy of 1,293 keV.

It is this isomer whose existence was inferred from indirect evidence by Ghiorso *et al.*¹². In their work, the ^{254}No ions were produced in a fusion-evaporation reaction and captured in a He gas jet, which deposited them on the surface of a wheel. The wheel was stepped past a number of detection stations, which recorded the characteristic 8.1 MeV α -decays from ^{254}No . They also noticed the detection of 8.1 MeV α -decays in detectors that were no longer adjacent to an irradiated spot on the wheel. From this they deduced the existence of an isomeric state with $T_{1/2} = 280 \pm 40\ \text{ms}$, which decays via the emission of a high energy ($\sim 1\ \text{MeV}$) γ -ray. They argued that this γ -ray imparts sufficient recoil momentum to the ^{254}No ion to allow it to leave the surface of the wheel for subsequent

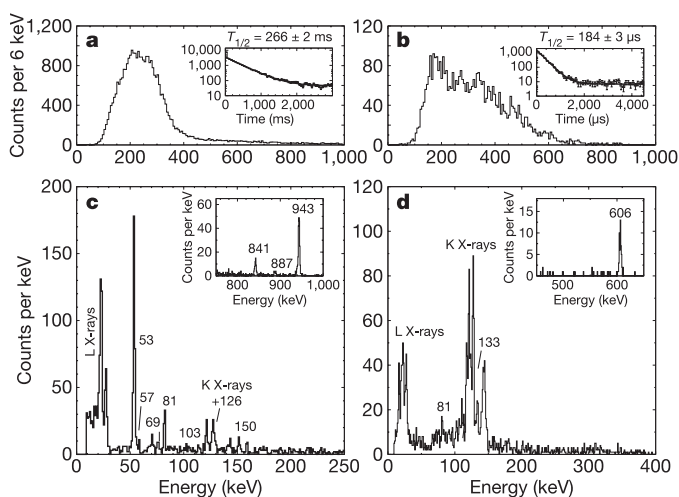


Figure 2 | Experimental data illustrating the decay of the two observed isomeric states. **a, b**, Main panels, the calorimetric electron signals from the two isomer decays; insets, the time distribution between the recoil implantation and the decay of the isomer. The different decay paths result in different energy signatures with endpoint energies of $\sim 400\ \text{keV}$ for the 266 ms isomer and $\sim 600\ \text{keV}$ for the 184 μs isomer. **c, d**, Main panels, the γ -rays observed in prompt coincidence with these electron signals. The difference in the ratio of the K and L X-rays is helpful for unravelling the decay paths. Insets, the high-energy regions of the spectra.

deposition onto the surface of a detector. The subsequent α -decay of the ground state could then be recorded independent of the position of the wheel. The present half-life of $T_{1/2} = 266 \pm 2$ ms agrees very well with the previous value¹². In addition, the high energy γ -rays (841 and 943 keV) observed in this work can provide the recoil momentum to the ²⁵⁴No ions necessary to explain the tentative findings of Ghiorso *et al.*¹² and thus provide a solution for a 30-year-old experimental puzzle.

Tantalizing first glimpses of a short-lived isomer were seen in our earlier experiments²⁴, but owing to low statistics we were unable to deduce a half-life or a decay scheme. In the present work, the short-lived 184 μ s isomer has been unambiguously identified. It feeds the 266 ms isomer via a cascade of transitions that include a prominently visible 606 keV γ -ray (Fig. 2d). Other experimental signatures essential to untangling the complex decay path of this isomer are given by the rather large total energy emitted in the form of conversion and Auger electrons (Fig. 2b) and the presence of strong L and K X-rays, which clearly show that the majority of these transitions are highly converted and therefore do not proceed via γ -ray emission. Taking into account the measured total energy, this isomer must lie at least 1.2 MeV higher in excitation energy than the 266 ms isomer. We have also modelled the decay path of this isomer to determine a lower limit on the spin of this state, and find the best agreement of the calorimetric electron signal if one assumes a decay path that changes the total angular momentum by 6–8 units, suggesting a spin of $J \geq 14$ for this isomer.

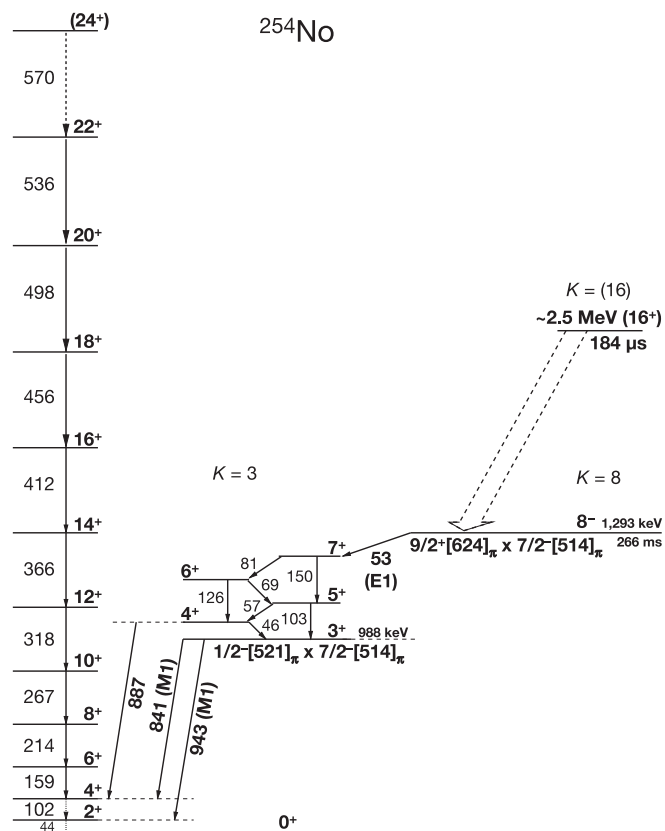


Figure 3 | Proposed level scheme of ²⁵⁴No. The 266 ms 8^- isomer is connected to the ground state via an excited 3^+ two-quasi-particle $\{1/2^- [521]_\pi \times 7/2^- [514]_\pi\}^{(3^+)}$ band. The 184 μ s (16^+) isomer populates the 8^- isomer band. Details of the exact decay path are tentative, but include a prominent 606 keV γ -ray and a cascade of conversion electrons, which establish its position in the level scheme. Transitions are labelled by their energies in keV, J^π denotes the spin and parity of a level. Levels are grouped according to the projection of the total angular momentum on the symmetry axis of the nucleus (K quantum number).

From the single-particle orbitals predicted around the Fermi surface, both a two-neutron structure $\{7/2^+ [624]_v \times 1/2^+ [620]_v\}^{(3^+)}$ and a two-proton structure $\{1/2^- [521]_\pi \times 7/2^- [514]_\pi\}^{(3^+)}$ with spin and parity 3^+ can be formed. (Here states are identified via the standard Nilsson labels of the single quasi-particles in question.) To distinguish between these configurations experimentally, the g -factors of both these configurations can be calculated from the g -factors of the individual orbitals to give $g_K^{vv} = 0.530$ and $g_K^{\pi\pi} = 0.824$. The experimental value of $g_K^{\text{exp}} = 0.87 \pm 0.14$ clearly identifies the $K = 3$ band head as a two-proton excitation involving the $1/2^- [521]_\pi$ orbital, stemming from the spherical $2f_{5/2}$ orbital above the $Z = 114$ shell. Thus it is now possible for theoretical models to use this firm assignment as a stepping stone to constrain the parameterizations used in the prediction of the spherical superheavy nuclei.

A unique feature of this mass region is the occurrence of both two-proton and two-neutron high- K isomers with spin and parity $J^\pi = 8^-$ at low excitation energies. The structure of the 266 ms isomer has been calculated by a number of authors^{11,12,25,26}. A two-quasi-proton state with a structure $\{7/2^- [514]_\pi \times 9/2^+ [624]_\pi\}^{(8^-)}$ is found in all calculations. The low-lying isomeric two-quasi-neutron state is generally calculated with structures $\{7/2^+ [624]_v \times 9/2^- [734]_v\}^{(8^-)}$ and $\{7/2^+ [613]_v \times 9/2^- [734]_v\}^{(8^-)}$. In all calculations, the structures are predicted to lie at excitation energies between 1 and 2 MeV. We compare the predictions and recent calculations (using the projected shell model^{27,28}) with the experimental data in Fig. 4. The majority of calculations consistently predict the two-neutron configuration to lie lowest in energy, although the two-proton configuration should be favoured for the 8^- state over the two-neutron configurations, as the decay from the isomeric $\{7/2^- [514]_\pi \times 9/2^+ [624]_\pi\}^{(8^-)}$ state to the $\{1/2^- [521]_\pi \times 7/2^- [514]_\pi\}^{(3^+)}$ state only involves the transition of a single proton rather than that of two protons and two neutrons. However, the long half-life of the isomer might be partially due to just such a change. This highlights the problems faced by experimenters if they have to rely on guidance from theory to make structural assignments in this region: theoretical calculations still cover a wide range, and it is precisely the kind of detailed data presented here that will have a major impact on the development of these models.

The structure of the 184 μ s isomer has not been discussed in the literature so far. We propose that this state is built on a

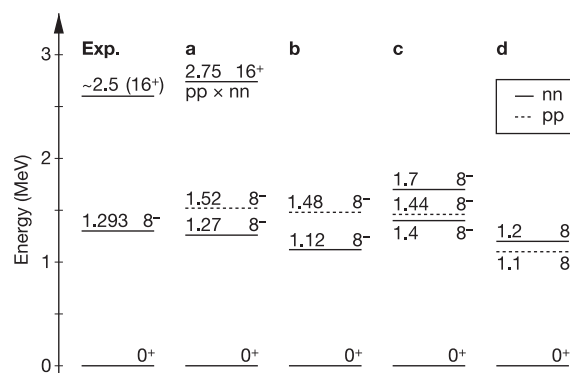


Figure 4 | Suggested configurations of the 266 ms isomer. The lowest configurations are the two-neutron $\{7/2^+ [624]_v \times 9/2^- [734]_v\}^{(8^-)}$ (a) and $\{7/2^+ [613]_v \times 9/2^- [734]_v\}^{(8^-)}$ (b–d) two-quasi-particle configurations with spin and parity $J^\pi = 8^-$. The two-quasi-proton $\{7/2^- [514]_\pi \times 9/2^+ [624]_\pi\}^{(8^-)}$ configuration lies higher in energy in nearly all cases. Levels are labelled with energy in MeV and the Nilsson labels of the individual quasi-particle levels. The indices v and π as well as the labels nn and pp refer to neutron and proton configurations, respectively. Exp, experimental levels deduced in this work. a, Calculations from this work (Y.S.); b, ref. 11; c, ref. 25; and d, ref. 26.

two-proton two-neutron four-quasi-particle configuration. One attractive choice is the $\{[7/2^- [514]_{\pi} \times 9/2^+ [624]_{\pi}]\}^{(8^-)} \times \{7/2^+ [624]_{\nu} \times 9/2^- [734]_{\nu}\}^{(8^-)}\}^{(16^+)}$ state calculated to lie at 2.75 MeV; that is, the product of the two-proton and two-neutron choices for the 8^- isomer, analogous to the well-known 16^+ isomer²⁹ in ^{178}Hf . This assignment is favoured by projected shell model calculations, but it must be stressed that this assignment on experimental grounds alone is highly tentative, and may change as the experimental situation improves.

The central result of our work is the firm experimental assignment of a state in the $Z = 102$ nucleus ^{254}No involving the crucial $2f_{5/2}$ proton orbital at the heart of the question of shell-stabilized superheavy elements. We have shown experimental evidence for two isomeric states in ^{254}No , determined their half-lives and decay paths and assigned two- and four-quasi-particle configurations to them. Similar two-quasi-particle high- K isomeric states are expected in neighbouring nuclei, for example, ^{252}No (ref. 30) and $^{248,250}\text{Fm}$ (ref. 12). The production cross-sections for these nuclei are lower than for ^{254}No , but the prospect of establishing a systematic picture of the dominant quasi-particle energies in this region is essential to arrive at an understanding of superheavy elements. The states studied in this work provide the most stringent test cases so far for the predictive power of model calculations in the deformed $Z \approx 100$ region.

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LETTERS

Magnetic impurity formation in quantum point contacts

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A quantum point contact (QPC) is a narrow constriction between two wider electron reservoirs, and is the standard building block of sub-micrometre devices such as quantum dots and qubits (the proposed basic elements of quantum computers). The conductance through a QPC changes as a function of its width in integer steps of $G_0 = 2e^2/h$ (where e is the charge on an electron, and h is Planck's constant), signalling the quantization of its transverse modes^{1,2}. But measurements of these conductance steps also reveal an additional shoulder at a value around $0.7G_0$ (refs 1–4), an observation that has remained a puzzle for more than a decade. It has recently been suggested^{5,6} that this phenomenon can be explained by the existence of a magnetic ‘impurity’ in the QPC at low electron densities. Here we present extensive numerical density-functional calculations that reveal the formation of an electronic state with a spin-1/2 magnetic moment in the channel under very general conditions. In addition, we show that such an impurity will also form at large magnetic fields, for a specific value of the field, and sometimes even at the opening of the second transverse mode in the QPC. Beyond explaining the source of the ‘0.7 anomaly’, these results may have far-reaching implications for spin-filling of electronic states in quantum dots and for the dephasing of quantum information stored in semiconductor qubits.

A QPC is usually formed by applying a negative voltage to a split gate (Fig. 1a), depleting the electrons in the two-dimensional electron gas (2DEG) under it to form a narrow and short constriction connecting large, two-dimensional regions of 2DEG. The number of occupied quantized transverse modes can be changed as a function of the applied gate voltage. As each mode contributes G_0 to the conductance (owing to spin degeneracy), the conductance rises in steps quantized at integer multiples of G_0 (refs 1, 2). A magnetic field lifts the spin degeneracy, leading to steps in multiples of e^2/h . Surprisingly, many experiments observe, at zero magnetic field, an additional shoulder near $0.7G_0$, a feature usually referred to as the 0.7 anomaly^{3,4}, which merges smoothly with the $0.5G_0$ plateau in a large magnetic field. Although less robust, an analogous anomaly was observed in the transition to the second conductance plateau at about $1.7G_0$ (ref. 4). A similar conductance structure was also found at large magnetic fields near crossings of spin-up and spin-down modes of different sub-bands⁷.

There have been several attempts to explain the 0.7 anomaly in terms of an antiferromagnetic Wigner crystal⁸, spontaneous sub-band splitting^{9–11}, or by assuming that the QPC supports a local quasi-bound state⁶. In the last case, as one electron is transported through this state, Coulomb interactions suppress the transport of an electron with opposite spin, reducing the conductance to around $0.5G_0$. The Kondo effect—the screening of this local spin by the conduction electrons—enhances the conductance at low temperatures towards G_0 . Indeed, experiments⁵ reveal features characteristic of the Kondo effect and the continuous evolution of the conductance

from $0.7G_0$ at higher temperatures to G_0 at low temperatures. But how can a QPC, being an open system, support a quasi-bound state? Previous numerical investigations report conflicting results^{12–14}. Below we present detailed spin-density-functional theory¹⁵ (SDFT) calculations showing that such a local moment can indeed form as the conductance of a QPC rises towards the first plateau. Additionally we present evidence that the formation of a quasi-bound state may also lead to the observed 1.7 anomaly and the anomaly at the crossing of sub-bands in large magnetic fields.

The set-up we used in our calculation is shown in Fig. 1b. In order to make the calculation tractable, we modelled the reservoirs as semi-infinite quantum wires. They are wide enough to carry many modes and thus resemble well the two-dimensional reservoirs in experimental set-ups. Using SDFT within the local spin-density approximation, we calculated the spin densities of the 2DEG and the charge distribution on the electrodes self-consistently. The nonlinearity of the equations may lead to several stable solutions that differ in their energy (details of our numerical approach are given in the Methods section). Here we present results for a specific QPC, as described in Fig. 2. However, the results are generic: we studied QPCs of lithographic length from 100 nm to 400 nm, of lithographic width from

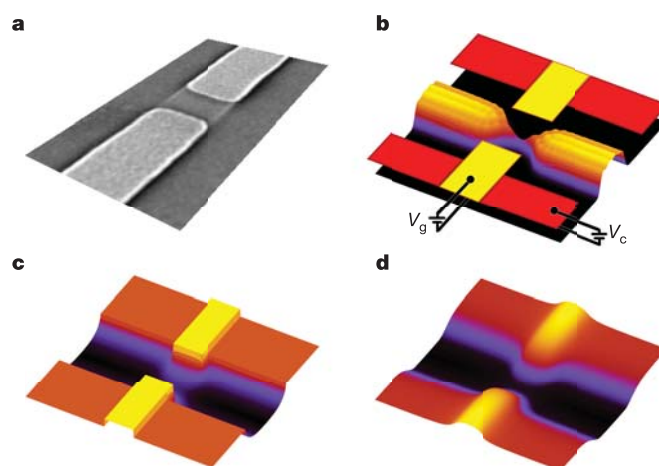


Figure 1 | Quantum point contact. **a**, Micrograph of a split gate forming a QPC. (Image from ref. 5, with permission.) **b**, The set-up used in the calculation. Voltage (V_g) applied to split gates (yellow) forms a QPC between two quantum wires defined by negatively biased (V_c) confining electrodes (red). Also shown is the density of the 2DEG near pinch-off where only one mode is occupied within the QPC, while the quantum wires carry several modes. The colour scale extends from zero (black) to $0.85 \times 10^{11} \text{ cm}^{-2}$ (yellow). **c**, **d**, Typical potentials in the plane of the electrodes (**c**) and in the 2DEG (**d**). Potentials on the electrodes are constant and are the input to the calculation, determining the effective potential in the plane of the 2DEG.

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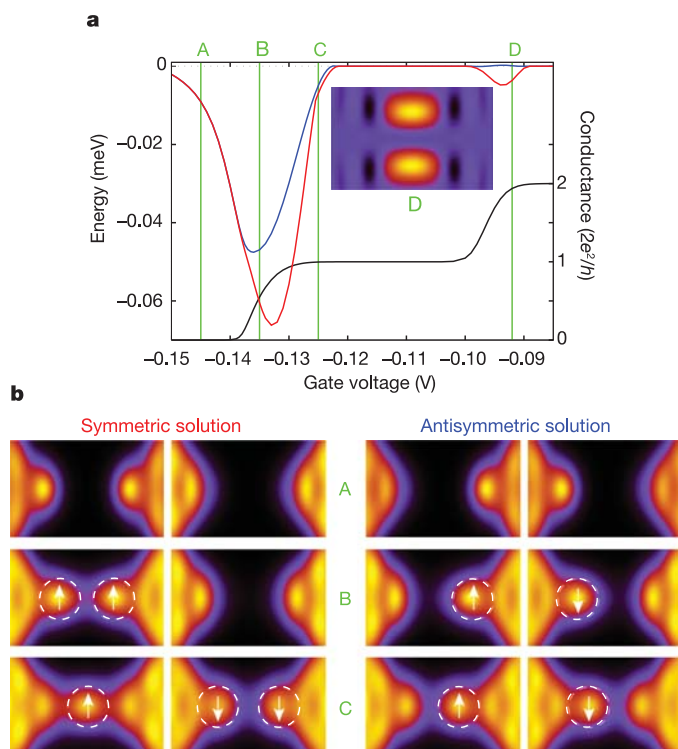


Figure 2 | States with spin polarization in a QPC. The QPC with a lithographic width and length of 200 nm and 250 nm, respectively, forms a constriction in a quantum wire with a lithographic width of 300 nm. The confining electrodes are biased to $V_c = -0.08$ V. The donor layer provides an electron density of 10^{11} cm^{-2} , and is 20 nm below the surface. The 2DEG forms 50 nm below the surface. **a**, Energies of the symmetric (red) and antisymmetric (blue) spin-polarized solutions relative to the energy of the unpolarized solution. Polarized solutions appear in the transition from pinch-off to the first plateau and then on the rise to the second plateau. The black line is a rough approximation to the conductance of the QPC, as calculated from the Kohn-Sham wavefunctions of the unpolarized solution (right-hand scale). Note, however, that for the lowest-energy, polarized solution the first conductance plateau will start around $V_g = -0.122$ V, on the right of point C. **b**, Spin densities of the symmetric and antisymmetric solutions for spin-up electrons (left columns) and spin-down electrons (right columns) at three values of gate voltage as indicated in **a**. The colour scale extends from zero (black) to $0.35 \times 10^{11} \text{ cm}^{-2}$ (yellow). A 400-nm-long and 100-nm-wide region about the centre of the QPC is shown. At A, the QPC is pinched-off; there are two polarized regions on each side of the QPC. At B, the two polarized regions begin to overlap. At C, in the symmetric solution the electrons in the QPC rearrange in such a way that a spin-1/2 magnetic moment forms. The inset to **a** shows spin polarization in the symmetric solution just below the second conductance plateau, at point D.

150 nm to 250 nm, and with 2DEG electron densities from 10^{11} to $2 \times 10^{11} \text{ cm}^{-2}$, with very similar results.

We first consider a QPC in the absence of a magnetic field. For gate voltages corresponding to conductance plateaus there is a unique solution to our equations, which shows no spin polarization. Although such a solution is also present in the regions between the plateaus, additional solutions exhibiting spin polarization in the QPC appear there. We classify these solutions according to their spatial symmetry: in the 'symmetric' and 'antisymmetric' solutions the polarization is respectively the same or opposite on the two sides of the QPC. In Fig. 2a, the energies of the polarized solutions relative to the energy of the unpolarized solution are plotted for gate voltages from pinch-off to the second conductance plateau. The symmetric solution, when present, is always the ground state of the system.

Figure 2b shows the evolution of spin densities of the two polarized solutions from pinch-off to the point where a spin-1/2 magnetic moment forms in the QPC. At point A the QPC is pinched-off: there

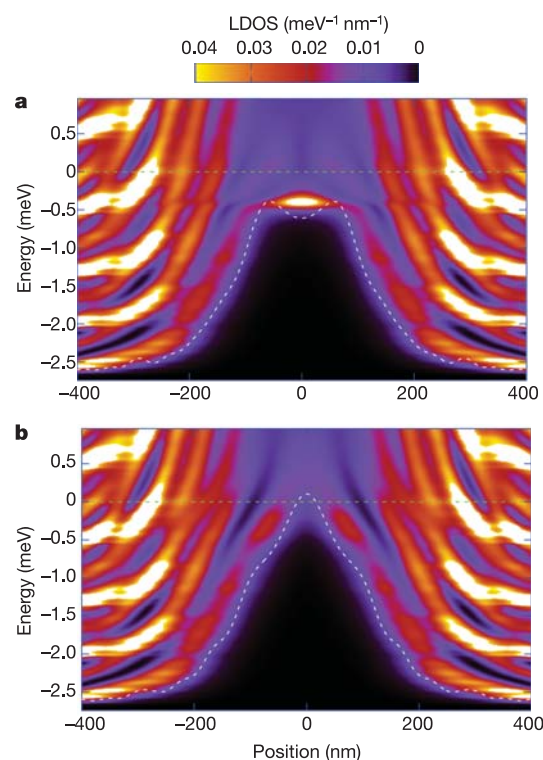


Figure 3 | Formation of a magnetic moment. **a**, Spin-up and **b**, spin-down local densities of states (LDOS), integrated over the cross-section of the QPC, for the QPC from Fig. 2 at $V_g = -0.125$ V. The Kohn-Sham potential for electrons in the lowest transverse mode is also shown (white dashed line). The potential for spin-up electrons has a double-barrier form near the centre of the QPC, and supports a quasi-bound state about 0.5 meV below the Fermi energy (green dashed line). Spin-down electrons also form quasi-bound states in the shoulders of the potential on both sides of the QPC. The bright stripes on both sides of the QPC correspond to the quasi-one-dimensional bands of the reservoirs.

is an extended region about the centre of the QPC where the density vanishes as the potential is much higher than the Fermi energy. The potential decreases towards the reservoirs and at some point it crosses the Fermi energy. Here are regions where the electron gas is polarized: the density is very low and the gain in (negative) exchange energy outweighs the additional kinetic energy incurred by polarization. The two polarized regions on both sides of the QPC do not overlap in this regime. The degeneracy of the ground state is thus fourfold: each of the polarized regions is spin-degenerate. With increasing gate voltage, the potential at the QPC gets lower and the electron density drifts inwards, forming narrow fingers that eventually reach the centre of the QPC (point B in Fig. 2). In the process, the polarized regions become increasingly decoupled from the reservoirs: two quasi-bound states, each corresponding to roughly one electron, form on each side of the QPC. As the potential barrier at the centre of the QPC gets weaker, the tunnelling probability through the barrier becomes appreciable and the conductance increases from zero. On increasing the gate voltage even further, a different configuration becomes energetically favourable in the symmetric subspace: a single electron forms a weakly coupled quasi-bound state at the centre of the QPC, accompanied by two electrons of opposite spin in regions further away towards the reservoirs (point C in Fig. 2). The degeneracy of the ground state is now twofold: the QPC acts as a spin-1/2 magnetic impurity. The spin-resolved local density of states (LDOS), that is, the number of states per energy and length interval (Fig. 3), provides additional insight into this state. The QPC potential for one of the spin components assumes a double-barrier form (due to Friedel oscillations), and a resonant state forms in its minimum.

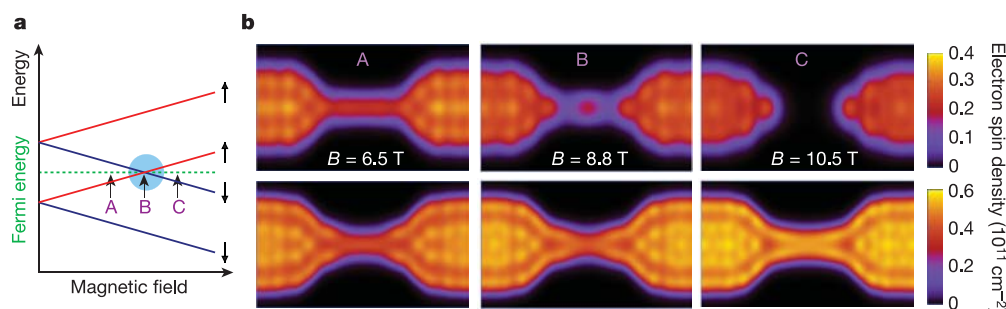


Figure 4 | Quasi-bound state in a large magnetic field. **a**, The Zeeman splitting of mode energies at the top of a QPC. The energy of the spin-up electrons in the lowest mode crosses the energy of the spin-down electrons in the higher mode at some value of the magnetic field. At a specific value of the gate voltage this crossing occurs near the Fermi energy. **b**, Spin-up (top row) and spin-down (bottom row) electron densities at three values of a magnetic field B near the degeneracy point. A 800-nm-long and 250-nm-wide area is

shown. At $B = 6.5$ T (point A in **a**), one mode is open for both spin orientations. At $B = 10.5$ T (point C), there are two open modes for spin-down electrons and none for spin-up electrons. In between, the bands cross and a quasi-bound state forms in the QPC ($B = 8.8$ T, point B). The QPC parameters are as in Fig. 2 except for the lithographic length, which is 300 nm. The gate voltage is $V_g = -0.102$ V.

The length of the QPC affects the formation of the spin-1/2 magnetic moment. In very short contacts, the transition to a well defined quasi-bound state does not take place at all: as the two polarized regions merge at the centre of the QPC, the conductance has already reached the first plateau. For longer contacts, the transition to the magnetic moment state shifts towards the pinch-off. In very long contacts, the configuration in Fig. 2b (symmetric solution at point C) evolves into an antiferromagnetically ordered chain.

The situation is somewhat similar on the rise from the first to the second conductance plateau: the density of electrons in the second mode is low there, and exchange again stabilizes states with a polarized QPC (Fig. 2a inset). This mechanism is not as efficient here as it was in the first mode: polarization in the second mode also induces (owing to exchange) partial polarization in the first mode. As the density in the first mode is large, there is a high kinetic energy cost involved. This is consistent with the experimental observation that not all QPCs exhibit the 1.7 anomaly.

In an external in-plane magnetic field, the energies of transverse modes for the two spin components split. The resulting polarized non-degenerate solution does not generally support a quasi-bound state. However, as shown in Fig. 4a, at a particular value of the field the energy of spin-up electrons (those with spin parallel to the field) in the first mode crosses that of spin-down electrons in the second mode. By tuning the gate voltage, one can also make the energy of the degeneracy point coincide with the Fermi energy. Figure 4b shows the evolution of spin densities with magnetic field in the vicinity of this crossing. At the degeneracy point a well-defined quasi-bound state forms, but, unlike the zero-field solution, it has a definite spin, determined by the field. This may be the source of the '0.7 analogues' observed at high magnetic fields⁷.

The formation of a local spin-degenerate quasi-bound state (supported by the extensive SDFT calculations presented here) is a necessary condition for the Kondo effect, which is beyond the local spin-density approximation used here. Interestingly, the calculations indicate that near pinch-off, two such states form on the two sides of the QPC. This may lead to the physics of the two-impurity Kondo model. Depending on the ratio of the coupling between these impurities, and their couplings to their respective reservoirs, one would expect to observe a zero-bias anomaly, with a split zero-bias peak, in this regime^{16,17}. The splitting should increase with increasing conductance. The formation of such polarized states at the QPC may also affect the spin-filling of quantum dots formed between two QPCs. Additionally, as quantum dots have been proposed as qubits (the building blocks of quantum computers), these degenerate quasi-bound states must be considered seriously—the degeneracy allows decoherence of quantum processes at very low temperatures. As short decoherence times will degrade the performance of any quantum computer, it will be necessary to ensure that the QPCs

forming the qubits are outside the quasi-bound-state formation regime.

METHODS

Model. We treated the 2DEG, electrodes and the donor layer as a set of three electrostatically coupled strictly two-dimensional systems. We assumed the donor layer was uniform and fully ionized. Then, according to spin-density-functional theory¹⁸, the properties of the system can be uniquely determined in terms of spin densities $n_{\uparrow}(\mathbf{r})$ and $n_{\downarrow}(\mathbf{r})$ of the 2DEG and by the distribution of charge on the electrodes $n_{el}(\mathbf{r})$ ($\mathbf{r}$ is the position in either the plane of the 2DEG or the plane of the electrodes). In particular, the energy of the system is a functional of the densities:

$$E[n_{\uparrow}, n_{\downarrow}, n_{el}] = E_{es}[n_{\uparrow}, n_{\downarrow}, n_{el}] + T_s[n_{\uparrow}, n_{\downarrow}] + E_{xc}[n_{\uparrow}, n_{\downarrow}] + \frac{1}{2}g\mu_B \int d\mathbf{r} B(\mathbf{r})[n_{\uparrow}(\mathbf{r}) - n_{\downarrow}(\mathbf{r})] - \sum_i N_i V_i$$

Here $E_{es}[n_{\uparrow}, n_{\downarrow}, n_{el}]$ is the electrostatic energy of the charge distribution. The presence of the semiconductor was taken into account through the use of the GaAs dielectric constant $\kappa = 12.9$ and a modified form of the Coulomb interaction, reflecting the dielectric mismatch at the surface¹⁹. We treated the 2DEG quantum-mechanically by including its kinetic energy (T_s) and exchange-correlation energy (E_{xc}) in the energy functional, taking into account that the effective mass of electrons in GaAs is 0.067 times the bare electron mass. We used the local spin-density approximation for the exchange-correlation functional, as parameterized in ref. 20. The fourth term in the energy functional is the Zeeman energy due to an in-plane magnetic field, with $g = 1.9$ for GaAs quantum wires⁷. Finally, as we compared the energies of different solutions at fixed voltages between electrodes and the 2DEG, we applied a Legendre transform to the energy functional (the last term in the expression above), with N_i and V_i being respectively the number of electrons and the voltage on i th electrode, and the sum running over all the electrodes in the system. The correct densities minimize the above functional subject to the applied voltages, and a constraint that the total number of electrons should match the charge provided by the donor layer.

Calculations. The minimization procedure yields Kohn-Sham equations¹⁵ for the 2DEG, with a constant electrostatic potential on each of the electrodes. In each iteration of the self-consistency loop, we first solved for the Kohn-Sham scattering states and calculated the density of the 2DEG. Using an iterative approach, we then redistributed the remaining electrons on electrodes in such a way that the potential there assumed the required form. In this step we performed the calculation on a large rectangular box, with periodic boundary conditions, which enabled us to employ the fast Fourier transform method and thus make the calculation efficient. We used the resulting charge distribution to calculate an improved electrostatic potential in the 2DEG. To obtain spin-polarized solutions at zero external magnetic field, we broke the symmetry by applying a magnetic field of an appropriate form (spatially symmetric or antisymmetric) in the initial iterations of the self-consistent procedure.

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Author Contributions Y.M. initiated the project. T.R. performed numerical simulations. T.R. and Y.M. analysed results and co-wrote the paper.

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LETTERS

Dependence of single-molecule junction conductance on molecular conformation

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Since it was first suggested¹ that a single molecule might function as an active electronic component, a number of techniques have been developed to measure the charge transport properties of single molecules^{2–12}. Although scanning tunnelling microscopy observations under high vacuum conditions can allow stable measurements of electron transport, most measurements of a single molecule bonded in a metal–molecule–metal junction exhibit relatively large variations in conductance. As a result, even simple predictions about how molecules behave in such junctions have still not been rigorously tested. For instance, it is well known^{13,14} that the tunnelling current passing through a molecule depends on its conformation; but although some experiments have verified this effect^{15–18}, a comprehensive mapping of how junction conductance changes with molecular conformation is not yet available. In the simple case of a biphenyl—a molecule with two phenyl rings linked by a single C–C bond—conductance is expected to change with the relative twist angle between the two rings, with the planar conformation having the highest conductance. Here we use amine link groups to form single-molecule junctions with more reproducible current–voltage characteristics¹⁹. This allows us to extract average conductance values from thousands of individual measurements on a series of seven biphenyl molecules with different ring substitutions that alter the twist angle of the molecules. We find that the conductance for the series decreases with increasing twist angle, consistent with a cosine-squared relation predicted for transport through π -conjugated biphenyl systems¹³.

We recently demonstrated that metal–molecule–metal junctions, formed by breaking Au point contacts in a solution of molecules, exhibit more reliable and reproducible conductance values when amine groups rather than thiols or isonitriles are used to attach the molecules to the junction contacts¹⁹. Because of this reduced variability, we can determine statistically meaningful average conductance values for specific single-molecule junctions; this capability in turn allows us to study the effect of molecular properties on junction conductance.

We present our experimental results as conductance histograms, where peaks indicate the most prevalent molecular junction conductances while the width of the conductance distributions reflects the microscopic variations from junction to junction. (For details of experimental and data analysis procedures, see Supplementary Information.) Figure 1a shows the histograms for 1,4-diaminobenzene (**1**) and 2,7-diaminofluorene (**2**), each constructed from thousands of conductance traces without resorting to any data selection or processing. All conductance traces reveal step-wise changes in conductance at conductance values that are close to multiples of the fundamental quantum of conductance, G_0 ($G_0 = 2e^2/h$, where e is the charge on an electron, and h is Planck's constant). In addition,

many traces also exhibit step changes below G_0 that are due to conduction through a single molecule bridging the gap between the two Au point-contacts (see Fig. 1c and Supplementary Fig. S1). As seen in the histograms of **1** and **2** (Fig. 1a), for each junction type the

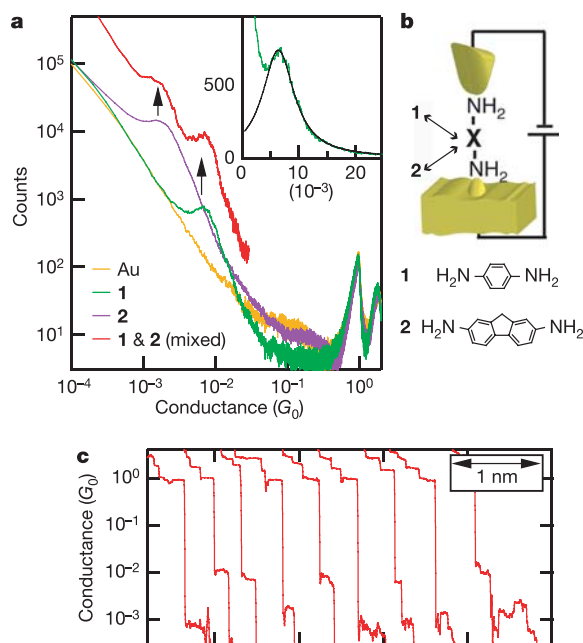


Figure 1 | Conductance measurements of 1,4-diaminobenzene and 2,7-diaminofluorene junctions. **a**, Conductance histogram of **1** (green; constructed from 10,000 traces and scaled by 1/10) and **2** (purple; constructed from 15,000 traces and scaled by 1/15) on a log–log scale, along with a control histogram of Au measured without molecules in the junction (yellow; constructed from 6,000 traces and scaled by 1/6). Peaks are clearly visible at G_0 and $2G_0$ for all three curves, and at $6.4 \times 10^{-3}G_0$ for **1** and at $1.5 \times 10^{-3}G_0$ for **2**. Also shown is a histogram collected for an equimolar solution of both molecules (vertically offset red curve; constructed from 4,000 traces and scaled by 1/4) showing two peaks indicated by the arrows corresponding to the individual peaks in the green and purple curve. Inset, Lorentzian fit to the sub- G_0 peak in the histogram of **1**; note that conductance and counts are now shown on a linear scale. The bin size is $10^{-3}G_0$ for all histograms, and data were collected using a fixed bias voltage of 25 mV. **b**, Top, illustration of a gap between a gold point-contact and a gold surface that can be bridged with either molecule **1** or **2** from the surrounding solution. Bottom, structure of **1** and **2**. **c**, Sample conductance traces obtained when pulling the point contacts apart, and exhibiting step-wise changes corresponding to the conductance of molecule **1** and **2**.

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additional step occurs within a narrow distribution of conductance values. The most prevalent value is determined by a fit to the peak below G_0 in the conductance histograms using a lorentzian line shape, which we find fits our peaks more accurately than a gaussian line shape (see Fig. 1a inset, and Supplementary Information).

When the experiment is repeated using a solution containing an equimolar mixture of **1** and **2** (as indicated in Fig. 1b), the resulting histogram (red curve in Fig. 1a) shows two distinct peaks below G_0 at nearly the same conductance values as those of the peaks seen in the histograms for the individual molecules (green and purple curves). Individual traces using the solution mixture show conductance plateaus corresponding to either molecule **1** or **2**, and sometimes a plateau corresponding to **1** followed by a plateau corresponding to **2** (Fig. 1c). But we do not see evidence of rapid fluctuations between the two, so the molecules are not exchanging position within the junction on the timescale of the experimental traces (few milliseconds for each trace). This provides further evidence that the conduction is through an individual molecule, not an ensemble.

Molecules **1** and **2** do not have an internal twist degree of freedom, but the two benzene rings of a biphenyl can rotate relative to one another. For such a molecule, as the twist angle (θ) between the two rings increases and simultaneously the degree of π -conjugation between them decreases, the junction conductance will decrease because molecular electron transfer rates scale as the square of the π -overlap^{14,20}. When neglecting the contribution of tunnelling transport through the σ -orbitals (which is within the error of our measurements), then theory predicts a $\cos^2\theta$ relation¹³. We probe this relation between molecule twist angle and conductance using seven different biphenyl molecules (molecules **2** to **8** listed in Table 1), where the size of the substituents on the proximal carbons is optimized to create structures with a twist angle ranging from flat to essentially perpendicular. Figure 2a shows the structure of four

(**2**, **4**, **6** and **8**) out of the seven molecules used. For each molecule, the average junction conductance is determined from the location of the peak in the conductance histogram, whereas the twist angle is determined theoretically for each molecule by assuming a fully relaxed and static geometry (see Table 1 and Supplementary Information; but effects of dynamic structural fluctuations of the molecule in the junction will also be discussed later on).

Figure 2b shows the measured conductance histograms for the molecules in Fig. 2a. For the flat molecule (**2**), the histogram peak yields an average junction conductance value of $1.5 \times 10^{-3} G_0$. For 4,4'-diaminobiphenyl (**4**) with equal molecular length but a twist angle θ of 34° , the conductance histogram shows a peak occurring at a lower conductance of $1.1 \times 10^{-3} G_0$. As the angle between the two aromatic rings is increased further to 52° in 4,4'-diaminooctafluorobiphenyl (**6**), the conductance value also drops further, to $4.9 \times 10^{-4} G_0$. When all four hydrogens on the proximal carbons of this molecule are replaced with methyls to give 2,6,2',6'-tetramethyl-4,4'-diaminobiphenyl (**8**) with a twist angle θ of about 88° , most counts in the histogram occur for low junction conductance values. In Fig. 2c, we plot the peak conductance value measured for the seven molecules against $\cos^2\theta$ and fit the data with a $\cos^2\theta$ curve (dashed line in Fig. 2c). The good fit indicates that the electronic effects of the substituents do not significantly alter the simple picture that junction conductance may be adjusted by simply decreasing the π -overlap between phenyl rings within the bridging molecule.

Further evidence for tunnelling conductance through these aromatic diamine junctions is provided by Fig. 3a, which compares the histograms for a series of oligophenyls (molecules **1**, **4**, **9**) with 1, 2 and 3 phenyl rings. The inset shows that the plot of junction conductance versus molecule length fits an exponential form with a decay constant of 1.7 ± 0.1 per phenyl ring. This behaviour is direct evidence for non-resonant tunnelling transport through the

Table 1 | Molecular structure and measured properties

Molecule number	Structure	Conductance (G_0)		Peak width*	Twist angle ($^\circ$)
		Measured	Calculated		
1		6.4×10^{-3}	6.4×10^{-3}	0.4	—
2		1.54×10^{-3}	2.1×10^{-3}	0.8	0
3		1.37×10^{-3}	2.2×10^{-3}	0.8	17
4		1.16×10^{-3}	1.6×10^{-3}	0.9	34
5		6.5×10^{-4}	1.2×10^{-3}	1.3	48
6		4.9×10^{-4}	7.1×10^{-4}	0.6	52
7		3.7×10^{-4}	5.8×10^{-4}	0.9	62
8		$7.6 \times 10^{-5}\ddagger$	6.4×10^{-5}	NA†	88
9		$1.8 \times 10^{-4}\ddagger$	3.5×10^{-4}	2.1	—

Table shows molecule structure, measured conductances, calculated relative conductances, relative widths of the histogram peaks (see Supplementary Information for details) and the calculated twist angle, θ .

* Half-width at half-maximum of the lorentzian fit, normalized to the peak value.

† The histogram peak was determined after subtracting the Au histogram from the data, as the raw data could not be fitted with a lorentzian so a width could not be determined.

‡ Determined from actual maximum of the raw data.

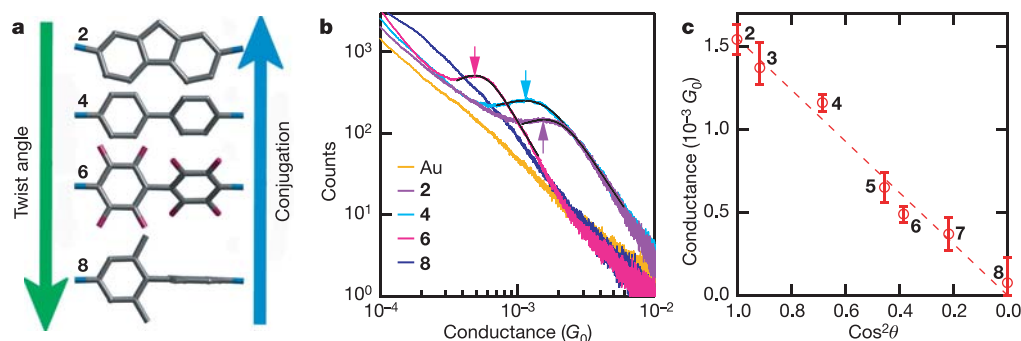


Figure 2 | Biphenyl junction conductance as a function of molecular twist angle. **a**, Structures of a subset of the biphenyl series studied, shown in order of increasing twist angle or decreasing conjugation. **b**, Conductance histograms obtained from measurements using molecule **2** (purple; constructed from 15,000 traces and scaled by 1/15), **4** (cyan; constructed from 7,000 traces and scaled by 1/7), **6** (pink; constructed from 11,000 traces and scaled by 1/11) and **8** (blue; constructed from 5,000 traces and scaled by 1/5). Also shown is the control histogram obtained from measurements

without molecules between the contacts (yellow; constructed from 6,000 traces and scaled by 1/6). Arrows point to the peak conductance values obtained from lorentzian fits (solid black curves). All data were taken at a bias voltage of 25 mV. **c**, Position of the peaks for all the molecules studied plotted against $\cos^2\theta$, where θ , the calculated twist angle for each molecule, is listed in Table 1. Error bars are determined from the standard deviation of the peak locations determined from the fits to histograms of 1,000 traces (see Supplementary Information.)

amine-terminated molecules^{14,20–24}, with the measured decay constant being close to the estimate of 1.5 per phenyl ring that we obtain^{25–27} for these molecules (see Supplementary Information). In Table 1, we also compare the measured conductance of all molecules with an estimated relative conductance, obtained by calculating the

square of the tunnel coupling of each molecule and normalizing this value to the measured conductance of molecule 1. This comparison indicates that the trend in the measured conductances is largely accounted for by the change in tunnelling with molecular conformation.

The results presented so far agree quite well with predictions of non-resonant tunnelling transport through static molecules. However, it is well established that the energy barriers for ring rotations in unsubstituted biphenyls such as **4** are about 0.1 eV or 2–3 kcal mol^{−1} (ref. 28; the barriers are altered by the substituents in the molecules **5–8**, and are substantially higher (Supplementary Information)). Therefore at room temperature, such molecules in the junction can be expected to fully explore low-energy rotations. The measurement times are long compared to the rate of molecular rotations in solution (each conductance step lasts a few milliseconds), so the conductance level measured at each step on a single trace is a thermal average over any fast dynamic fluctuations. But even though the rotation barrier is small for molecule **4**, our calculations indicate that thermal averaging still results in a conductance value that is close to the value characteristic of a static molecule in its lowest energy conformation.

The data in Figs 1–3 also reveal another interesting trend: as the molecule is granted additional rotational degrees of freedom, the conductance peak broadens. This is in contrast to the data for the diamino-alkane series¹⁹, where the histogram peak widths are similar for all molecules and are associated only with the modest variations in the electronic coupling through the Au–amine link. The peak broadening for the aromatics (widths in Table 1) is most clearly seen in Fig. 3a for the oligophenyl series. The sharpest peak is observed for **1**, while the peaks in the histograms are systematically broader for biphenyl- and terphenyl-diamines (**4** and **9**), where the individual phenyl rings can rotate about the σ -bond that connects them. Individual conductance traces show steps (Supplementary Figs S1 and S2) that occur over an expanded range. We therefore suggest that in each junction formed during the measurements, the molecule is in a different average conformation enabled by its rotational degrees of freedom and that each different conformation has a different characteristic conductance. In solution, the thermally averaged conformation for molecule **4** would have both the ring–ring twist angle and the two amine–ring twist angles close to their rotational barrier minima of 34° and 90° respectively (angles illustrated in Fig. 3b). Upon junction formation, each Au–N link is pinned by the particular Au–N bonding environment. This provides a mechanism to skew the twist angles of the trapped molecule. The average twist angles of the three rotatable bonds in the trapped molecule (the C–C and the two C–N σ -bonds) would then differ from those in the unbound molecule, with the bond that has a lower

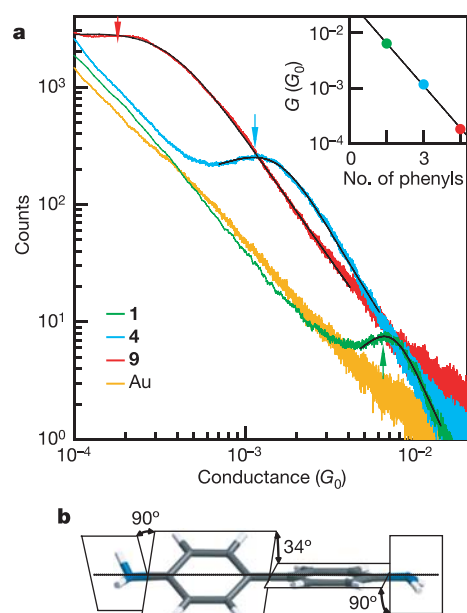


Figure 3 | Polyphenyl junction conductance as a function of the number of phenyl units. **a**, Conductance histograms for molecular junctions of **1** (green; constructed from 10,000 traces and scaled by 1/10), **4** (cyan; constructed from 7,000 traces and scaled by 1/7), and **9** (red; constructed from 3,000 traces and scaled by 1/3), along with a control histogram obtained from measurements without molecules between the contacts (yellow; constructed from 6,000 traces and scaled by 1/6). Bin size is $10^{-5}G_0$ for the green trace (and is therefore offset vertically by a factor of 10) and $10^{-6}G_0$ for the other traces. Arrows point to the peaks. Lorentzian fits (solid black curves) are also shown. All data were taken at 25 mV bias voltage. Inset, peak conductance values obtained with **1**, **4** and **9** versus the molecules' number of phenyl rings, on a semi-log scale. The black line shows an exponential fit to the data points. **b**, Illustration of molecule **4**, highlighting the three twist angles that influence conductance through the metal–molecule–metal junction, and which are defined as the angles between the adjacent planes shown in the figure. In solution, molecule **4** has a thermally averaged conformation with a ring–ring twist angle and amine–ring twist angles close to the potential minimum values of 34° and 90°, respectively.

energy barrier for rotation accommodating a larger change. This results in wider histograms, although the histogram peaks, which correspond to the most probable Au–molecule–Au conformations, are still located close to the lowest energy conformation.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed L.V. (latha@phys.columbia.edu) or M.S.H. (msh2102@columbia.edu).

LETTERS

Sulphur isotope evidence for an oxic Archaean atmosphere

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The presence of mass-independently fractionated sulphur isotopes (MIF-S) in many sedimentary rocks older than ~2.4 billion years (Gyr), and the absence of MIF-S in younger rocks, has been considered the best evidence for a dramatic change from an anoxic to oxic atmosphere around 2.4 Gyr ago^{1–9}. This is because the only mechanism known to produce MIF-S has been ultraviolet photolysis of volcanic sulphur dioxide gas in an oxygen-poor atmosphere. Here we report the absence of MIF-S throughout ~100-m sections of 2.76-Gyr-old lake sediments and 2.92-Gyr-old marine shales in the Pilbara Craton, Western Australia. We propose three possible interpretations of the MIF-S geologic record: (1) the level of atmospheric oxygen fluctuated greatly during the Archaean era; (2) the atmosphere has remained oxic since ~3.8 Gyr ago, and MIF-S in sedimentary rocks represents times and regions of violent volcanic eruptions that ejected large volumes of sulphur dioxide into the stratosphere; or (3) MIF-S in rocks was mostly created by non-photochemical reactions during sediment diagenesis, and thus is not linked to atmospheric chemistry.

The abundance ratios of the stable isotopes of sulphur (³²S, ³³S, ³⁴S and ³⁶S) in a material *i* are typically expressed as the deviations from those in troilite (FeS) in Cañon Diablo meteorite, such as $\delta^{33}\text{S}_i(\text{‰}) = [({}^{33}\text{S}/{}^{32}\text{S})_i/({}^{33}\text{S}/{}^{32}\text{S})_{\text{VCDT}} - 1] \times 1,000$, where VCDT is the international sulphur isotope standard. The $\delta^{33}\text{S}$ and $\delta^{34}\text{S}$ values of most Earth materials typically fall on the terrestrial fractionation line¹⁰: $\delta^{33}\text{S} = 0.515 \times \delta^{34}\text{S}$. Such relationships occur because the magnitude of isotope fractionation during most (bio)chemical reactions depends primarily on differences in isotope mass, which is termed mass-dependent fractionation. Deviation from the terrestrial fractionation line is commonly expressed as: $\Delta^{33}\text{S} = \delta^{33}\text{S} - 0.515 \times \delta^{34}\text{S}$. A $\Delta^{33}\text{S}$ range of $0 \pm 0.2\text{‰}$ has been suggested for mass-dependent fractionation^{5,11}; $\Delta^{33}\text{S}$ values outside this range reflect MIF-S.

Previous researchers^{1,5–9} have suggested a general trend with geologic time in $\Delta^{33}\text{S}$ values of sedimentary rocks (Fig. 1): Stage I (>2.4 Gyr), distinct MIF-S ($\Delta^{33}\text{S} = -2\text{‰}$ to $+8\text{‰}$); Stage II (~2.4 to ~2.0 Gyr), small or no MIF-S ($\Delta^{33}\text{S} = 0 \pm 0.5\text{‰}$); and Stage III (<2.0 Gyr), no MIF-S ($\Delta^{33}\text{S} = 0 \pm 0.2\text{‰}$). By irradiating SO₂ gas with an ultraviolet laser (wavelength $\lambda = 193$ nm) in the absence of O₂, Farquhar *et al.*³ produced S⁰ (elemental sulphur) with large positive $\Delta^{33}\text{S}$ values ($+65 \pm 5\text{‰}$) and SO₄^{2–} with large negative $\Delta^{33}\text{S}$ values ($-17 \pm 5\text{‰}$). From photochemical modelling, the maximum atmospheric *p*O₂ value to create MIF-S by ultraviolet photolysis of SO₂ was estimated to be ~10^{–2} present atmospheric level (PAL)^{3,5} or ~10^{–5} PAL (ref. 4). From these data sets, recent investigators^{1–9} have concluded that the following major changes occurred ~2.4 Gyr ago: (1) the atmosphere dramatically changed from anoxic (*p*O₂ < 10^{–5} PAL) to oxic (*p*O₂ > 10^{–5} or >10^{–2} PAL); (2) input of atmospheric S⁰ to the oceans seceded because of the

complete conversion of volcanic SO₂ to SO₄^{2–} in an oxic atmosphere; and (3) oceanic SO₄^{2–} concentration began to rise due to the increased oxidative weathering of pyrite.

In some Archaean (>2.5 Gyr) sedimentary rocks, very small $\Delta^{33}\text{S}$ values (within $0 \pm 0.2\text{‰}$) have been recognized^{1,5–8}. However, their significance cannot be fully evaluated without mineralogical and geochemical information to determine whether sulphur compounds recorded the isotopic signatures of the contemporaneous atmosphere or those of subsurface environments. Here we present the results of isotopic, mineralogical and geochemical investigations of modern weathering-free drill cores recently recovered by the Archaean Biosphere Drilling Project from two major Archaean sedimentary formations in the Pilbara Craton: the world's oldest lake sediments (the 2.76-Gyr-old Hardey Formation) and one of the oldest marine shales (the 2.92-Gyr-old Mosquito Creek Formation; Fig. 2). The Archaean Biosphere Drilling Project, geologic settings of the drill sites, and the methods and results of analyses are described in the Supplementary Information.

Sulphur occurs in our samples almost exclusively as pyrite (FeS₂) crystals, though not as detrital grains from the weathering of igneous rocks or sulphide-rich ore bodies, nor had they formed from hydrothermal fluids during and/or after sediment deposition. The morphology and modes of occurrence of pyrites in the Archaean shales are very similar to those in Devonian black shales, which suggests they grew from Fe-S precursors that formed from H₂S in the overlying water bodies and/or in sediment pore waters during sediment accumulation and diagenesis. Most (if not all) of the H₂S was probably generated by the reduction of aqueous SO₄^{2–} by sulphate-reducing bacteria.

Essentially all pyrites in Phanerozoic sediments were formed by sulphate-reducing bacteria that used the SO₄^{2–} and organic matter in water, which explains the general correlations between the concentrations of sulphide-S in sediments and SO₄^{2–} in water^{12,13}. For example, SO₄^{2–} concentration averages 28 mM in modern oceans and ~1 mM in freshwater lakes, which results in differing sulphide-S concentrations (~0.2 wt% versus ~0.05 wt%) and S-to-C weight ratios (~0.37 versus ~0.07) between average normal marine sediments (those deposited under oxygenated sea water) and average lake sediments. Because H₂S concentration in a euxinic water body is much higher than in an open sea, sediments deposited in such conditions generally have much higher pyrite contents (S = ~0.5 to ~10 wt%) and variable S/C ratios.

The S-to-C concentration relationships of the 2.76-Gyr lake sediments (Hardey Formation) are very similar to modern sediments in low-SO₄^{2–} lakes, as are those of the 2.92-Gyr-old marine shales (Mosquito Creek Formation) to modern sediments in euxinic seas (Fig. 3). This implies that the SO₄^{2–} concentrations of the 2.92-Gyr-old Mosquito Creek Sea were comparable to modern euxinic seas

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(~20 to ~30 mM)^{12,13} but much higher than modern lakes, and that sulphate-reducing bacteria were actively producing H₂S that resulted in pyrite formation. This suggestion contrasts with a current popular Archaean sulphur cycle model^{1-9,14} that postulates very-low-SO₄²⁻ oceans (<1/100 of the present level) and minimal sulphate-reducing bacteria activity. From the $\delta^{34}\text{S}$ values of pyrites (Supplementary Tables), we estimate the $\delta^{34}\text{S}_{\text{SO}_4}$ values were $\geq +2\text{‰}$ for the 2.76-Gyr-old lake and $\geq +8\text{‰}$ for the 2.92-Gyr-old sea, and the kinetic isotopic fractionation of $^{34}\text{S}/^{32}\text{S}$ accompanying sulphate reduction $\Delta_{\text{SO}_4-\text{H}_2\text{S}} = \delta^{34}\text{S}_{\text{SO}_4} - \delta^{34}\text{S}_{\text{H}_2\text{S}}$ was $\geq 5\text{‰}$ in the lake and $\geq 7\text{‰}$ in the sea.

Because the background concentrations of aqueous sulphur species in the Archaean Hardey Lake were probably much less than those in the Mosquito Creek Sea, we expected to recognize easily the contributions of atmospheric sulphur to the surface waters and to detect larger $\Delta^{33}\text{S}$ variations in the Hardey (lacustrine) samples. However, this was not so. The $\delta^{34}\text{S}$ and $\Delta^{33}\text{S}$ values of the 18 lacustrine samples range only from -2.73‰ to $+1.74\text{‰}$ and -0.21‰ to $+0.25\text{‰}$, respectively (Figs 1 and 4). With an analytical uncertainty of $\pm 0.05\text{‰}$ for $\Delta^{33}\text{S}$, we suggest that all these samples fall within the mass-dependent fractionation range. The $\delta^{34}\text{S}$ and $\Delta^{33}\text{S}$ values of 24 marine shale samples show slightly larger variations, from $+1.40\text{‰}$ to $+7.7\text{‰}$ and -0.51‰ to $+0.18\text{‰}$, respectively; all but five samples fall within the mass-dependent fractionation range. Typical sedimentation rates for shales are ~1 to ~10 cm per

1,000 years (ref. 15), so our data suggest that during the ~1- to ~10-Myr period required to accumulate the ~100-m section of non-marine Hardey (or marine Mosquito Creek) shales, the surface waters did not receive measurable contributions of atmospheric S⁰ or SO₄²⁻ with MIF-S signatures.

Large MIF-S values were previously recognized in sedimentary rocks 2.47–2.72 Gyr and 3.2–3.8 Gyr in age (Fig. 1). Farquhar and Wing⁵ concluded that ~0.5% to ~10% of the sulphur used in pyrite formation during Stage I (>~2.4 Gyr) came from atmospheric S⁰, while the remainder (>90%) came from the bacterial sulphate reduction of sea water SO₄²⁻, which was mostly supplied by the oxidative weathering of pyrite on land. They also suggested the $\Delta^{33}\text{S}$ values of Stage II (~2.4 to ~2.0 Gyr) sediments indicated an atmospheric $p_{\text{O}_2} > 10^{-5}$ (or $> 10^{-2}$) PAL, which prevented new additions of atmospheric S⁰ and SO₄²⁻ with large MIF-S signatures to the oceanic SO₄²⁻ reservoir. The small MIF-S in Stage II sediments may have resulted from the weathering of older land sediments with MIF-S signatures into the oceans⁵. Previously, $\Delta^{33}\text{S}$ analyses were not reported on sedimentary rocks of a well-defined age between ~2.72 Gyr and ~3.0 Gyr. The $\Delta^{33}\text{S}$ values of the 2.76-Gyr-old Hardey and 2.92-Gyr-old Mosquito Creek formations are essentially the same as for Stage II pyrites (Fig. 1). Therefore, a scenario similar to that for Stage II sediments may be applicable to these formations. This would imply that atmospheric p_{O_2} levels fluctuated greatly (a 'yo-yo atmosphere') during the Archaean, possibly from anoxic

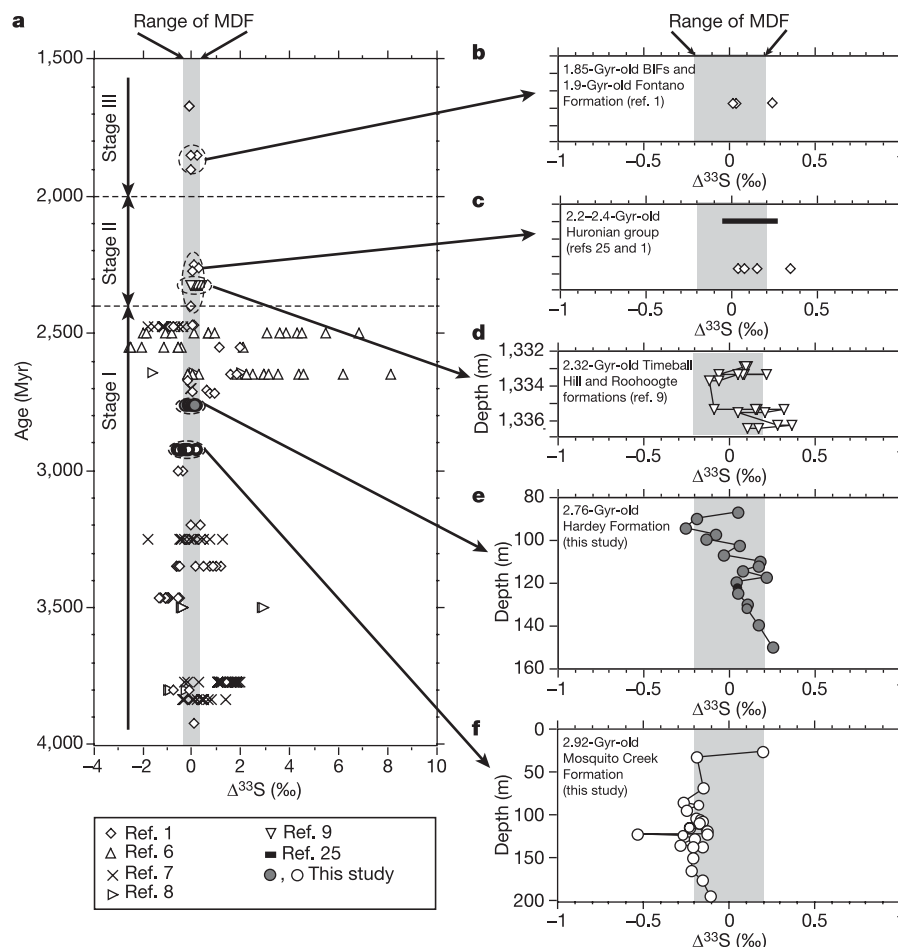


Figure 1 | MIF-S record. $\Delta^{33}\text{S}$ values of sulphur-bearing compounds (mostly pyrite and/or barite) in pre-1.6-Gyr-old sedimentary rocks. Each point represents one analysis. $\Delta^{33}\text{S}$ values outside the shaded range ($\Delta^{33}\text{S} = 0 \pm 0.2\text{‰}$) may contain MIF-S atoms. **a**, A summary of data from the literature and this study. Sources of data and methods of analysis: refs 1, 6 and 25, SF₆ method using bulk rock/sulphide sulphur converted Ag₂S; refs 8 and 9,

spot analyses of pyrite crystals using SF₆ method; ref. 7, spot analyses of single sulphide grains by secondary ion mass spectrometry. The divisions of stages I–III are from ref. 5. **b–f**, Plots of $\Delta^{33}\text{S}$ values versus the stratigraphic positions of samples. The 1.85-Gyr-old BIFs in **b** refer to the Biwabik and Gunflint banded iron formations. The thick bar in **c** is the range of isotope values from ref. 25.

Age	Group	Formation	Myr
Proterozoic	Hammersley group	Boolgeeda Iron	2,410
		Woongara volcanics	2,449
		Weeli Wolli	2,454
		Brockman Iron	2,470
		Mt McRae shale & Mt Sylvia	~2,500
		Wittenoon	2,542
		Carawine dolomite	2,590
		Marra Mamba Iron	2,593
		Jeerinah	2,630
		Maddina	2,690
Archaean	Mount Bruce supergroup	Maddina	2,717
		Tumbiana	2,720
		Kylena	2,740
		Hardey	2,760
		Mt Roe basalt	2,775
		Mosquito Creek	2,905
		Coondamar	2,926
		Pilbara supergroup	>3,000
		Pilbara supergroup	>3,000
		Pilbara supergroup	>3,000

Figure 2 | Stratigraphy of the Pilbara Craton. Stratigraphic column with ages comprising sedimentary volcanic rocks in the Pilbara-Hammersley districts, Western Australia^{26–28}. The five sedimentary formations (Mt McRae shale, Carawine, Jeerinah, Hardey and Mosquito Creek) subjected to sulphur isotope investigations by ref. 6 (dotted border) and this study (solid border) are outlined.

(<10^{−5} PAL) before ~3.0 Gyr, to oxic (>10^{−5} or >10^{−2} PAL) between ~3.0 and ~2.75 Gyr, to anoxic between ~2.75 and ~2.4 Gyr, back to oxic after ~2.4 Gyr. The recent discovery of molecular fossils of cyanobacteria and eukarya in 3.2-Gyr-old sediments from the Pilbara Craton¹⁶ supports such a scenario, because it suggests the possible development of an oxic atmosphere before ~2.5 Gyr ago. Future research on different geologic formations may suggest the atmospheric *p*_{O₂} level fluctuated even more frequently and rapidly during the Archaean.

Alternatively, the $\delta^{33}\text{S}$ record of sedimentary rocks (Fig. 1) may suggest that the atmosphere has remained oxic since ~3.8 Gyr ago, and that MIF-S signatures represent times and regions of violent volcanic eruptions that ejected large volumes of SO₂ to the stratosphere, where it would be above the ozone shield and hence subject to ultraviolet photolysis. The discovery of significant MIF-S signatures ($\Delta^{33}\text{S} = +0.67\text{‰}$ and -0.50‰) in volcanic ashes associated with the recent violent eruptions of Mt Pinatubo and another unknown volcano, and the absence of MIF-S in ashes associated with minor eruptions¹⁷, supports this interpretation. In fact, the 2.5-Gyr-old McRae and 2.7-Gyr-old Jeerinah shales, which exhibit large MIF-S signatures (Figs 1 and 4), contain abundant volcanogenic sediments¹⁸. The frequent occurrence of Archaean rocks with large MIF-S, but the decreasing magnitude of MIF-S in younger rocks, may be because Earth's interior was hotter and volcanic activity was much more intensive and extensive during the Archaean, as indicated by the abundance of Archaean komatiites, layered intrusions, granitoids and mantle plumes^{19,20}. In fact, the Archaean volcanic flux of SO₂ is estimated to be three to four times higher than today²¹.

The above arguments were made under the fundamental assumption

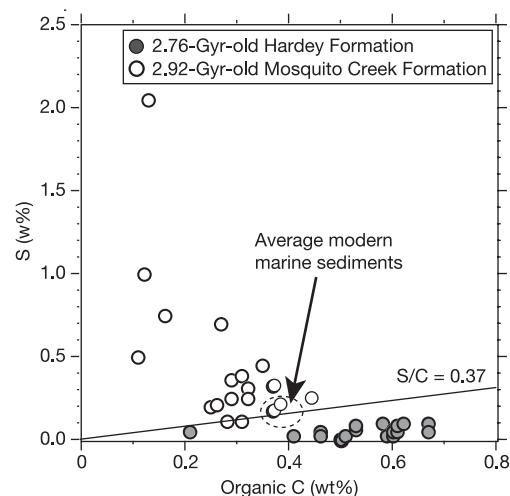


Figure 3 | S and organic C. S concentration versus organic C concentration for the studied samples, compared to average modern marine sediments^{12,13}.

tion that the only mechanism to create MIF-S signatures is the atmospheric photochemical reaction of volcanic SO₂. However, this assumption must be evaluated for the following reasons: (1) a serious discrepancy exists between photochemical experimental results and geologic measurements. Ultraviolet photolysis of SO₂ generates S⁰ with very large negative $\delta^{34}\text{S}$ ($-60 \pm 20\text{‰}$) and positive $\Delta^{33}\text{S}$ ($+65 \pm 5\text{‰}$) values³, while Ono *et al.*⁶ suggested that pyrite with the most positive $\delta^{34}\text{S}$ ($\sim +10\text{‰}$) and positive $\Delta^{33}\text{S}$ ($\sim +8\text{‰}$) values in the McRae and Jeerinah shales represented atmospheric S⁰ (Fig. 4). If so, the transformation of S⁰ with negative $\delta^{34}\text{S}$ (experimentally produced S⁰) to pyrite-S with positive $\delta^{34}\text{S}$ would have required an unidentified non-photochemical reaction in water and/or sediments that caused very large negative MIF-S effects ($\Delta^{33}\text{S} = \sim -70\text{‰}$). (2) By recognizing a significant difference in temperature dependence of the equilibrium fractionation factors for the ³³S–³²S and ³⁴S–³²S exchanges between S₂ and H₂S gases, Deines²² has shown that the $\Delta^{33}\text{S}$ values of S₂ and H₂S may range from infinitely large positive to infinitely large negative values at

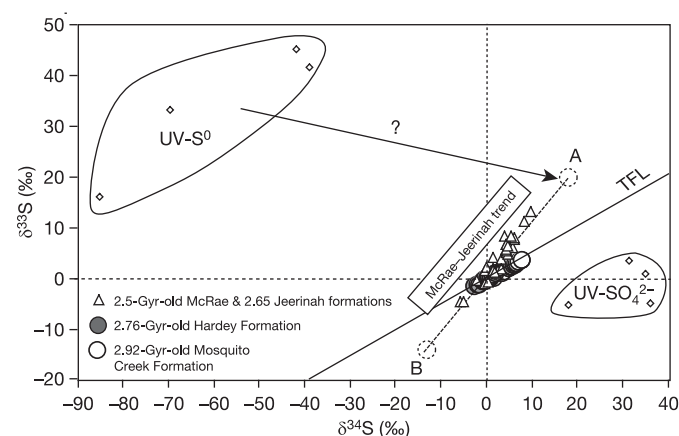


Figure 4 | $\delta^{33}\text{S}$ versus $\delta^{34}\text{S}$ relationships. $\delta^{33}\text{S}$ and $\delta^{34}\text{S}$ values of the 2.76-Gyr-old Hardey and 2.92-Gyr-old Mosquito Creek formations (this study) are compared to those of the 2.65-Gyr-old Jeerinah and the 2.5-Gyr-old Mt McRae shale formations⁶, and those of S⁰ (UV-S) and SO₄^{2−} (UV-SO₄^{2−}) produced by ultraviolet photolysis of SO₂ (ref. 3). Ono *et al.*⁶ interpreted the McRae-Jeerinah trend as the result of mixing two types of pyrite, one that formed from atmospheric S⁰ (A) and another from atmospheric SO₄^{2−} (B). However, the transformation of UV-S⁰ to atmospheric S⁰ (A) requires a non-photochemical reaction with large negative MIF-S effects (see text).

temperature $T \approx 71.5^\circ\text{C}$. (3) Watanabe *et al.*²³ were able to generate H_2S with distinct MIF-S signatures ($\Delta^{33}\text{S} = 0.30\text{--}0.45\text{‰}$) through the thermochemical reduction of SO_4^{2-} by amino acids at $T \approx 170^\circ\text{C}$. Combining these data with the Rayleigh and/or recycling sulphate-sulphide processes, they explained much larger $\Delta^{33}\text{S}$ values in sedimentary rocks. (4) Ono *et al.*²⁴ discovered distinct MIF-S signatures ($\Delta^{33}\text{S} = 0.27\text{--}0.34\text{‰}$) in pyrite crystals from a fossilized Jurassic ammonite (~ 150 Myr in age) that must have formed by the bacterial or thermochemical reduction of porewater SO_4^{2-} , by using organic matter in the ammonite during sediment diagenesis.

Therefore, it may be premature to assume that all MIF-S in rocks indicate an anoxic atmosphere. A more complete record of $\Delta^{33}\text{S}$ in Archaean and younger rocks, which includes the geologic, mineralogical and geochemical context, along with laboratory experiments on the fractionations of multiple sulphur isotopes during a variety of non-photochemical reactions, is required to relate the $\Delta^{33}\text{S}$ rock record to the atmospheric, thermal, and biological history of the Earth.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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LETTERS

Parochial altruism in humans

Helen Bernhard¹, Urs Fischbacher¹ & Ernst Fehr^{1,2}

Social norms and the associated altruistic behaviours are decisive for the evolution of human cooperation^{1–9} and the maintenance of social order¹⁰, and they affect family life, politics¹¹ and economic interactions¹². However, as altruistic norm compliance and norm enforcement often emerge in the context of inter-group conflicts^{13,14}, they are likely to be shaped by parochialism¹⁵—a preference for favouring the members of one's ethnic, racial or language group. We have conducted punishment experiments¹⁶, which allow 'impartial' observers to punish norm violators, with indigenous groups in Papua New Guinea. Here we show that these experiments confirm the prediction of parochialism. We found that punishers protect ingroup victims—who suffer from a norm violation—much more than they do outgroup victims, regardless of the norm violator's group affiliation. Norm violators also expect that punishers will be lenient if the latter belong to their social group. As a consequence, norm violations occur more often if the punisher and the norm violator belong to the same group. Our results are puzzling for evolutionary multi-level selection theories based on selective group extinction^{2–5} as well as for theories of individual selection^{17–19}; they also indicate the need to explicitly examine the interactions between individuals stemming from different groups in evolutionary models.

The human species is unique in the extent to which it regulates social life with normative obligations that constrain selfish behaviour^{1,20}. Social norms such as food sharing, or those related to cooperative hunting and participation in warfare, shaped human life throughout important evolutionary phases^{1,21–23}. It is therefore likely that the existence of these norms had a deep impact on the properties of human altruism, because norm obedience and norm enforcement involve important altruistic behaviours. The fact that social norms are group level phenomena²⁴ suggests that parochial social instincts^{15,25}—which we define as preferences for favouring the members of one's own social group—may have shaped human altruism in decisive ways. Norms emerge through interactions in groups and apply to interactions within groups; group members enforce them, and they often arise in the context of inter-group conflicts^{13,14}. Normative obligations are thus likely to apply only to ingroup members; people who do not belong to the group neither obey the norm nor benefit from the altruistic behaviours the norm enforces.

In this paper, we study the potentially parochial nature of altruistic norm enforcement by conducting third-party punishment experiments¹⁶ with members of two small, distinct, cohesive and non-hostile indigenous groups in the Western Highlands of Papua New Guinea (PNG)—the Wolimbka and the Ngenika. Centralized institutions for the enforcement of legal rules are largely absent in PNG, meaning that social norms almost exclusively regulate social life. In addition, PNG societies more closely resemble the human societies under which our social instincts evolved than the modern, complex societies in which most people at present live. PNG is therefore an ideal environment for studying the parochial nature of human altruism.

The use of cohesive, indigenous groups from simple societies distinguishes our study from minimal group experiments^{26,27} based on artificially created laboratory groups of students. Researchers have found some forms of ingroup favouritism in simple allocation experiments based on the minimal group paradigm. However, the ingroup favouritism observed in these experiments^{26,27} does not reveal parochial tendencies in human altruism because the subjects only distributed resources between two other subjects. Subjects thus bore no cost, regardless of how they allocated the available resources between the other two subjects. Moreover, subsequent research^{28,29} has shown that costless ingroup favouritism in these experiments is due to the expectation that ingroup members will receive some reciprocation from other ingroup members. Thus, what looked like a preference for ingroup favouritism was in fact based on the expectation of ingroup reciprocity²⁹.

In our study, we conducted an anonymous, one-shot, third-party punishment experiment¹⁶ involving a dictator (player A), a recipient (player B) and a third party (player C). Player A receives an endowment of 10 Kina (K), which is equivalent to a high daily labourer's wage in the informal sector of the economy. Player B, the recipient, has no endowment and player C, the third party, receives K5. First, A can transfer any amount between K0 and K10 to B. Then C is informed about A's transfer and has the opportunity to punish A's action by spending K0, K1 or K2 on punishment. Every Kina spent on punishment reduces A's income by K3. After player A and C had chosen their actions, we also elicited their expectations about how the dictators would be punished at the three transfer levels K0, K5 and K10.

Our experiment is designed to capture the altruistic enforcement of egalitarian sharing norms that ethnographic studies have documented^{22,30,31}. Such sharing norms are beneficial for the group because they insure group members against the uncertainties in individual food acquisition success. If an egalitarian sharing norm exists, we should observe both that dictators transfer money to the recipients and that third parties exhibit altruistic punishment⁷ for transfer levels below the equal split. As we wanted to examine the parochial behavioural patterns, we allocated each subject in our study to one of the following four treatment conditions. (1) All three players in the game are from the same tribe (treatment ABC). (2) Only players B and C are from the same tribe, while A is an outgroup member (BC). (3) Only players A and B are from the same tribe (AB). (4) Only players A and C are from the same tribe (AC). The decision-makers in all four treatments were informed about the other two players' group affiliation.

Current evolutionary models based on the idea that human altruism evolved through the selective (cultural or biological) extinction of groups in inter-group conflicts^{2–5} predict the following punishment pattern. No punishment should be observed in treatment AC if A does not share, because there is no obligation to share with a recipient B from the outgroup. Sharing with an outgroup member would only help the competing group, possibly at the

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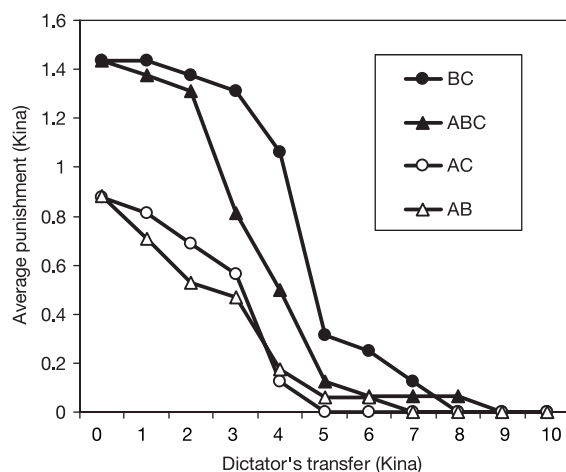


Figure 1 | Punishment pattern across conditions in the third-party punishment game. The third party spends money (Kina) on punishing the dictator for the violation of an egalitarian sharing norm in all conditions. Punishment expenditures by third parties increase if dictators reduce transfers below the egalitarian sharing norm; punishment is highest in all conditions if the dictator does not share at all. Punishment expenditures are much higher in the ABC condition, where dictator (A), recipient (B) and third party (C) belong to the same group, than in the AB condition (where only the dictator and the recipient belong to the same group) and the AC condition (where only the dictator and the third party belong to the same group). Contrary to predictions, punishment is also much higher in the BC condition (where the recipient and the third party belong to the same group) than in the AB and the AC condition.

expense of the ingroup. The same punishment prediction is made for condition AB, where the third party is an outgroup member; in this case A violates a sharing norm if she or he does not share with B, but player C—being an outgroup member—has no obligation to punish a norm violation within the other group. Likewise, the group competition theories predict zero punishment in condition BC because A is an outgroup member who is not obliged to obey a sharing norm in interactions with an ingroup member. Thus, the ABC treatment is the only condition in which evolution should have favoured altruistic punishment, because punishment in ABC sustains group norms that enhance a group's ability to compete with other groups.

We observe that the punishment pattern is qualitatively similar in all four treatment conditions (Fig. 1). There is little punishment for transfers at and above the egalitarian level, while sharing decisions that give the dictator a larger share of the 'pie' are more heavily punished the more the dictator deviates from the equal split. For example, even in the conditions with relatively low punishment levels (AB and AC), 58% of the third parties punish if the dictator transfers nothing, but only 3% punish at K5 and nobody punishes at transfers higher than K6. This finding suggests the existence of an egalitarian sharing norm in all four conditions, and not just in the ABC condition—a fact that is puzzling in view of the predictions above. The third parties' and the dictators' beliefs further support this interpretation. Regardless of the treatment condition, individuals in both roles believe that a transfer of K0 will be punished severely, while transfers of K5 or K10 will not be punished.

Although punishment of low transfers is not zero in the AB and the AC conditions, the size of the punishment across the ABC, the AC and the AB condition obeys the order that selective extinction models^{2–5} predict: punishment is much higher when all three players belong to the same group (ABC) compared to the AC and the AB treatments, where punishment is roughly the same (Fig. 1). The difference between ABC and the other two treatments is highly significant (ordered probit regression, $P = 0.007$, two sided, $N = 539$), while the difference between the AC and the AB conditions

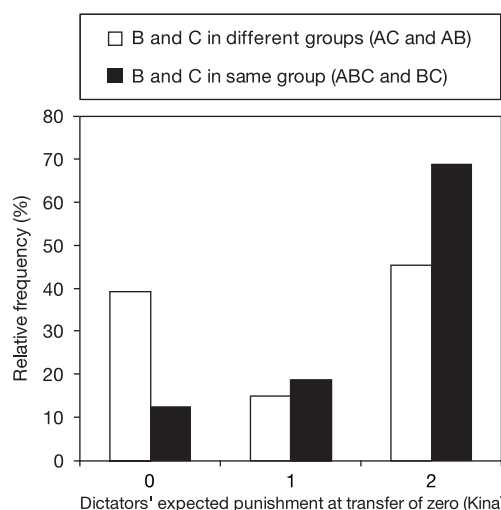


Figure 2 | The punishment threat for the lowest transfer level as perceived by the dictators. The punishment threat subjectively perceived is important, because higher expectations about punishment induce more compliance with the sharing norm. In those conditions where the recipient (B) and the third party (C) belong to the same group (that is, in conditions ABC and BC), dictators perceive a higher punishment threat than in conditions where B and C belong to different groups (that is, in conditions AB and AC).

is negligible and insignificant (ordered probit regression, $P = 0.947$, two sided, $N = 539$; see also statistical methods in Supplementary Information).

Punishment in the BC condition is also much higher than in the AC and the AB conditions (Fig. 1; ordered probit regression, $P < 0.001$, two sided, $N = 539$). Controlling for the transfer level, the probability that the third party punishes a violation of the sharing norm is 30.4 percentage points higher in the BC condition than in the AC and AB conditions. In fact, punishment in BC is even significantly higher than in ABC (Fig. 1; ordered probit regression, $P = 0.022$, two-sided, $N = 352$). The difference between these two conditions is particularly large at transfer levels of K3 and K4. For example, the average punishment at a transfer level of K4 is more than twice as high in BC than in ABC. Thus, third parties are more lenient if the norm violator belongs to their group.

These findings imply that regardless of the dictator's group affiliation, punishment is much higher if the recipient and the third party belong to the same group. Thus, to the extent to which third-party punishment deters potential norm violators, the victims of potential norm violations are much more protected by the threat of third-party punishment if the third party belongs to the victim's group. The dictators' expectations also support this deterrence effect of third-party punishment. The dictators expect significantly more punishment at K0 in those conditions where B and C belong to the same group (Fig. 2; ordered probit regression, $P = 0.019$, two sided, $N = 65$), and the expected punishment level at K0 has a significantly positive effect on transfer levels. A one-unit increase in expected punishment raises the transfer level by 1.43 units (tobit regression, $P < 0.001$, two-sided, $N = 65$). Thus, both actual punishment and punishment expectations suggest that victims of norm violations are much more protected if the victim and the third party belong to the same group.

How do these punishment patterns, together with the parochial pattern of voluntary norm compliance, shape the transfer levels? We find that the transfers are higher in those conditions where A and B belong to the same group (tobit regression, $P = 0.018$, two-sided, $N = 65$). The dictators even transfer more money in these conditions if we control for their punishment expectations (tobit regression, $P = 0.086$, two-sided, $N = 65$). Thus, dictators who expect the same

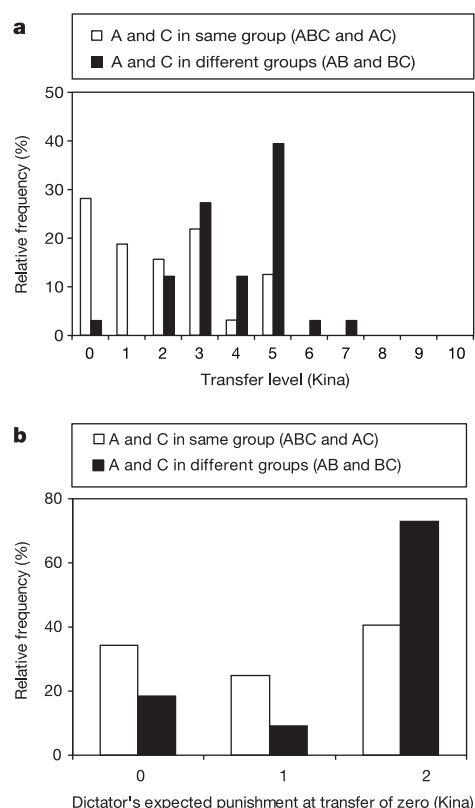


Figure 3 | Dictators' transfers and perceived punishment threats if the third party belongs to the dictator's group. a, Relative frequency of dictators' transfer decisions. Dictators comply much less with the egalitarian sharing norm if the third party belongs to their group. **b**, Relative frequency of dictators' perceived punishment threats in the case of a transfer of zero. Dictators believe that they will be punished much less if the third party belongs to their group.

level of punishment give more in those conditions where A and B belong to the same group, suggesting a higher degree of voluntary norm compliance in these treatments (see also statistical methods in Supplementary Information).

Player A's reluctance to share if B does not belong to his group is strongly reinforced if the third party comes from A's group. In this case the dictators' transfers are substantially lower on average compared to the conditions where A and C belong to different groups (Fig. 3a; tobit regression, $P < 0.001$, two-sided, $N = 65$). Moreover, the punishment threat subjectively perceived is significantly lower if the third party belongs to the dictator's group (ordered probit regression, $P = 0.022$, two sided, $N = 65$). For example, only 41% of the dictators expect the maximal punishment at a transfer of K0 if the third party is from their group, but 73% of the dictators expect the maximal punishment if the third party is from the other group (Fig. 3b). Thus, dictators expect that 'their' third parties will be lenient, inducing them to transfer little to the recipient.

Although our findings are puzzling from the viewpoint of current selective extinction models²⁻⁵, they also suggest how the models could be extended in order to explain the full empirical pattern. First, these models focus on norm enforcement within groups for the purpose of winning inter-group conflicts while neglecting the potential benefits from cooperative inter-group interactions. This approach makes it difficult to understand when hostility characterizes inter-group reactions and when cooperative norms govern them. The current models therefore have problems in explaining our first main result—the existence of egalitarian sharing norms in all four conditions—but a suitably extended model, which explicitly formalizes individual strategies in inter-group encounters, may be able to

capture this fact. Second, the lack of explicit modelling of individual inter-group encounters makes it also difficult to understand why—regardless of the norm violator's group affiliation—punishment is so high in those conditions where the third party and the recipient (ABC and BC) belong to the same group. Punishing outsiders who harm an ingroup victim increases the general security of all ingroup members by preventing attacks by outgroup members. If a group has a reputation for punishing individual attacks by outgroup members, the latter are deterred from such attacks and all ingroup members enjoy more protection. Thus, taking the problem of group reputation into account could possibly explain the high punishment in both the BC and the ABC conditions.

Current individual selection models¹⁷⁻¹⁹ also cannot readily explain the full pattern of punishment behaviours. Models based on repeated interactions or reputation formation^{9,18,19} seem to predict that punishment will be highest in the ABC condition, because the third party's reputational benefits from punishing are most favourable in this condition: protecting an ingroup victim may yield a future ally and punishing an ingroup violator lessens the likelihood of being cheated in future interactions with the norm violator. Kin selection theory¹⁷ is also not fully satisfactory, because the average genetic relatedness between two randomly selected adult tribe members in tribes of several hundred people is rather low, due to migration and marriages with outgroup members. It is therefore difficult to see why kin selection should have favoured a sharing norm that applies to all tribe members alike—in particular in situations such as our experiment, where the dictator's fitness loss roughly equals the recipient's fitness gain. Perhaps, if a sharing norm already exists for other reasons, kin selection might have favoured a lower punishment of ingroup members, which could explain the lower punishment in ABC compared to the BC condition.

Thus, if future research confirms the robustness of our results, the parochial patterns of human altruism constitute a challenge for existing evolutionary theories. Currently, no single theory seems to be able to explain the entire pattern of parochialism across treatments, providing an opportunity for developing new theories or modifying existing ones.

METHODS

Subjects. A total of 195 members—aged 17 to 60—of two small-scale societies in the Western Highlands of PNG (the Wolimbka and the Ngenika) participated in a third-party punishment game, permitting us to conduct 65 games with three players each. We conducted 17 games in the AB treatment and 16 in each of the other treatments.

Tribal warfare is a frequent event in PNG, but the Ngenikas and the Wolimbkas never conducted tribal warfare with each other within the memorized history of the older members of the two tribes. They are neutral towards each other and do not exchange any gifts or goods, except in the rare case of inter-tribe marriage. Due to the absence of any hostilities between the two tribes, finding parochialism across these two tribes makes our results even stronger.

Experimental procedures. Game instructions and procedures are based on the work of Henrich *et al.*³². In each experimental session, 18 participants first received some preliminary verbal instructions as a group. We ensured that the participants did not communicate about the game before the experiment. Each participant received a show-up fee of K3 and drew a number at the beginning. One at a time, in the order of the pulled numbers, the subjects then came into a separate room to participate in the experiment. The game was then explained to them verbally in much detail. Participants who failed to understand the instructions were dismissed from the experiment but could keep their show-up fee.

We elicited player C's punishment decision with the strategy method (see Supplementary Information). This means that player C indicated how much he or she is willing to spend on punishment for each of player A's feasible transfers. Player C made this decision before (s)he knew the dictator's actual transfer level. Since we collected 11 punishment decisions from each player C—one punishment decision for each feasible transfer level—we always controlled for repeated measurement in the statistical analysis of punishment decisions.

Statistical methods. The punishment decisions were examined with ordered probit regressions. We controlled for repeated individual measurements and for individuals' transfer levels in all regressions. Treatment effects were measured by dummy variables that take on a value of one if the observation comes from the

treatment of interest. Otherwise the dummy variable is zero. The dictators' transfer decisions were analysed with tobit regressions that consider the transfer level as a function of the expected punishment and the treatment dummies of interest.

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LETTERS

Mutations in progranulin cause tau-negative frontotemporal dementia linked to chromosome 17

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Frontotemporal dementia (FTD) is the second most common cause of dementia in people under the age of 65 years¹. A large proportion of FTD patients (35–50%) have a family history of dementia, consistent with a strong genetic component to the disease². In 1998, mutations in the gene encoding the microtubule-associated protein tau (MAPT) were shown to cause familial FTD with parkinsonism linked to chromosome 17q21 (FTDP-17)³. The neuropathology of patients with defined MAPT mutations is characterized by cytoplasmic neurofibrillary inclusions composed of hyperphosphorylated tau^{3,4}. However, in multiple FTD families with significant evidence for linkage to the same region on chromosome 17q21 (D17S1787–D17S806), mutations in MAPT have not been found and the patients consistently lack tau-immunoreactive inclusion pathology^{5–12}. In contrast, these patients have ubiquitin (ub)-immunoreactive neuronal cytoplasmic inclusions and characteristic lentiform ub-immunoreactive neuronal intranuclear inclusions^{11–13}. Here we demonstrate that in these families, FTD is caused by mutations in progranulin (PGRN) that are likely to create null alleles. PGRN is located 1.7 Mb centromeric of MAPT on chromosome 17q21.31 and encodes a 68.5-kDa secreted growth factor involved in the regulation of multiple processes including development, wound repair and inflammation¹⁴. PGRN has also been strongly linked to tumorigenesis¹⁴. Moreover, PGRN expression is increased in activated microglia in many neurodegenerative diseases including Creutzfeldt–Jakob disease, motor neuron disease and Alzheimer's disease^{15,16}. Our results identify mutations in PGRN as a cause of neurodegenerative disease and indicate the importance of PGRN function for neuronal survival.

FTD is characterized by abnormalities in behaviour, personality and language with relative preservation of perception and memory, and may also be associated with motor dysfunction, including both motor neuron disease (MND) and parkinsonism^{1,17}. The microscopic pathology of FTD varies markedly¹⁸; although some cases (~40%) have tau-positive neuronal inclusions, the majority lack tau-based histopathology and show ub-immunoreactive neurites and neuronal cytoplasmic inclusions (NCI). The ub-immunoreactive inclusions are characteristically found in layer II of the frontal and temporal neocortex and in the dentate fascia of the hippocampus (frontotemporal lobar degeneration with ubiquitin inclusions, FTLD-U)^{1,17}. This same pattern of ub-immunoreactive pathology

is also found in patients with MND and dementia¹⁹. Other than being ubiquitinated, the molecular composition of the NCI is presently unknown.

Previous genetic linkage studies in FTD families revealed a locus on chromosome 17q21²⁰ (Fig. 1a). Subsequently, over 30 mutations in MAPT (encoding tau) were identified that account for a proportion of these cases (FTDP-17)^{3,4} (<http://www.molgen.ua.ac.be/FTDmutations>). FTDP-17 patients with MAPT mutations inevitably develop tau neurofibrillary inclusion pathology⁴. However, since the identification of MAPT mutations in 1998, nine FTD families have been conclusively linked to chr17q21 but lack defined MAPT mutations (tau-negative FTD-17)^{5,12,13}. In each family, affected patients lack tau inclusions but develop ub-immunoreactive pathology typical of FTLD-U^{5–9,11–13}. Moreover, several of these families also have ub-immunoreactive lentiform neuronal intranuclear inclusions (NII) with a similar distribution to the NCI (Fig. 2a–c)^{5,6,11–13}. We previously found NII in other families—too small for genetic linkage analysis—with tau-negative FTD however these lesions were uncommon in sporadic FTD²¹. These findings led us to propose that NII are characteristic of FTD associated with this genetic locus^{12,21}.

The absence of obvious MAPT mutations in tau-negative FTD-17 families suggested that this form of FTD is caused either by novel MAPT mutations that have eluded detection or by mutations in a different gene. However, extensive sequencing of MAPT intronic and regulatory regions in two linked FTD-17 families (UBC17 and 1083), and analysis of soluble tau from patient brain extracts (UBC17), previously failed to find evidence of pathogenic MAPT mutations^{12,22}. Therefore, we examined other candidate genes within the 3.53-centimorgan (6.19-Mb) critical region defined by haplotype analysis in reported families (D17S1787–D17S806)¹². The coding exons of candidate genes were first sequenced in affected and unaffected members of UBC17 (Table 1), a large Canadian tau-negative FTD family with highly significant evidence for linkage to chromosome 17q21 (two-point LOD score of 3.65)¹². Analysis of over 80 genes (out of ~165 in the region) failed to identify a pathogenic mutation. However, when progranulin (PGRN) was sequenced, an insertion mutation of 4 base pairs (bp) was detected in exon 1 (c.90_91insCTGC) causing a frameshift at codon 31 that introduces a premature termination codon after a read through of 34 residues (C31LfsX34). PGRN is a 593-amino-acid (68.5-kDa) multifunctional growth factor that is composed of seven-and-a-half tandem

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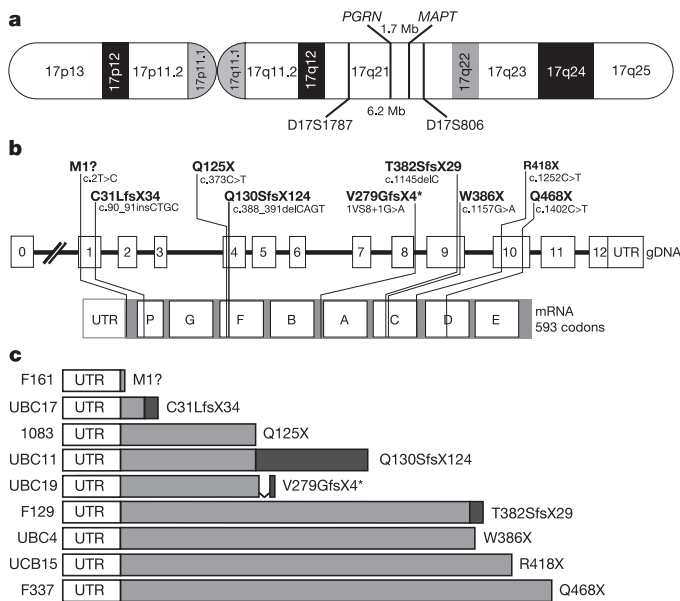


Figure 1 | Null mutations in *PGRN* cause tau-negative FTD linked to chromosome 17. **a**, Schematic representation of chromosome 17. *PGRN* is located 1.7 Mb from *MAPT*, which is mutated in FTDP-17. **b**, Schematic representation of the *PGRN* gene and mRNA encoding the PGRN protein, with positions of tau-negative FTD-17 mutations indicated. Lettered boxes refer to individual granulin repeats. **c**, Location of the mutation altering the Met 1 codon, and the premature termination codons created by the truncating mutations, in *PGRN* mRNA. The mutant mRNAs are subject to nonsense-mediated decay resulting in null alleles. The IVS8+1G>A mutation (indicated by an asterisk) is predicted to cause skipping of exon 8 (V279GfsX4).

repeats of a 12-cysteine granulin motif¹⁴. The mutant (C31LfsX34) *PGRN* protein is predicted to be only 65 residues in length, including the signal peptide, and does not contain a single intact cysteinyl repeat (Fig. 1). The C31LfsX34 mutation segregated with disease in UBC17 (Table 1, Supplementary Fig. 1), and was absent in 550 North American control individuals.

PGRN was then sequenced in affected individuals from an additional 41 families with clinical and pathological features consistent with tau-negative FTD. Families were identified in Canada (7 families), the USA (8 families), the UK (17 families), the Netherlands (1 family) and Scandinavia (8 families). This analysis identified an additional seven *PGRN* mutations in eight families, each predicted to cause premature termination of the coding sequence (Table 1, Fig. 1c). The mutations include four nonsense mutations (Q125X, W386X, R418X

and Q468X), two frameshift mutations (Q130SfsX124, T382SfsX29) and a mutation in the 5' splice site of exon 8 (IVS8+1G>A; in UBC19). The latter is likely to lead to skipping of exon 8 from the *PGRN* messenger RNA, resulting in a frameshift (V279GfsX4); however, this could not be confirmed as a source of RNA was not available. The Q130SfsX124 mutation was found in two FTD families ascertained independently in Canada (UBC11) and the UK (F53). All seven mutations segregated with disease in the relevant families (Table 1, Supplementary Fig. 1) and were absent in 200 North American and 95 UK controls.

The only two FTD families with significant evidence for linkage to 17q21 (UBC17¹² and 1083^{5,23}) were both found to have mutations in *PGRN* (Fig. 1, Supplementary Fig. 2). Notably, patients from all nine families with *PGRN* mutations met clinical criteria for FTD¹, without signs of MND, and all had neuropathological findings that included ub-immunoreactive NII, as predicted by our hypothesis that these lesions are a characteristic feature of FTD linked to this locus^{12,21}. Analysis of a further series of FTD families from Belgium also identified mutations in *PGRN*, including another family (DR8) with published evidence for linkage to chromosome 17q21^{11,23}.

To investigate the pathogenic mechanism of these truncating mutations, we determined whether premature termination of the *PGRN* coding sequence resulted in nonsense-mediated decay (NMD) of the mutant mRNAs²⁴. Quantitative PCR with reverse transcription (qRT-PCR) on RNA extracted from patient lymphoblasts carrying the R418X (UBC15) and C31LfsX34 (UBC17) mutations showed that both were associated with a ~50% reduction in total *PGRN* mRNA relative to lymphoblasts from unaffected individuals (Fig. 3a). In addition, *PGRN* mRNA from both families consisted almost entirely of wild-type mRNA with little of the mutant mRNAs detected (Fig. 3c, Supplementary Fig. 3b). Treatment of patient lymphoblasts with cycloheximide—a known inhibitor of NMD²⁴—resulted in an increase in levels of total *PGRN* mRNA (Fig. 3b) that was associated with a selective increase in the C31LfsX34 and R418X mutant mRNAs (Fig. 3c, Supplementary Fig. 3c). Furthermore, western blot analysis showed that wild-type *PGRN* protein was reduced in extracts from R418X and C31LfsX34 lymphoblasts (mean reduction 34%, $P = 0.01$, t -test) relative to extracts from unaffected relatives (Fig. 3d). We were also unable to detect the predicted mutant proteins in extracts from patient brain tissue (not shown) and lymphoblastoid cells (Fig. 3d). These data suggest that the observed premature termination mutations in *PGRN* cause disease by creating null alleles, with the mutant mRNAs being degraded by NMD. This results in loss of functional *PGRN* (haploinsufficiency) and presumably explains why there is no relationship between the location of each mutation and clinical phenotype, as each mutation has the same effect—creation of a null allele.

The proposed haploinsufficiency mechanism is also consistent

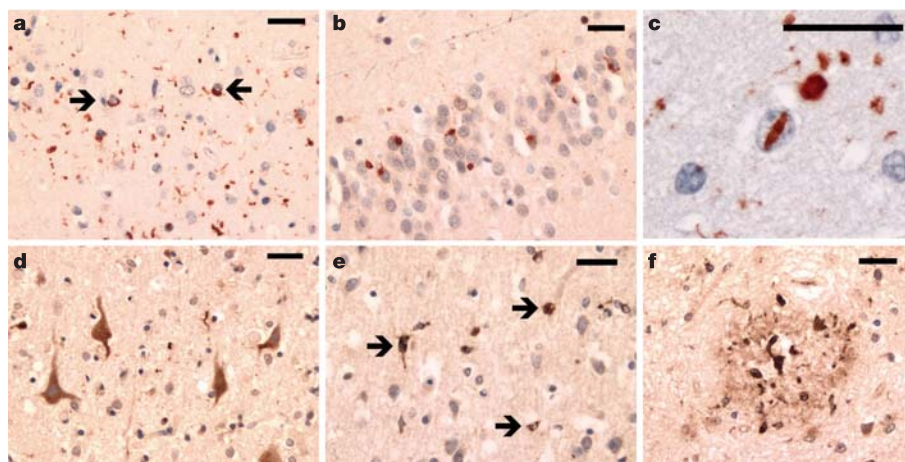


Figure 2 | Immunohistochemistry in FTD with *PGRN* mutations. **a–c**, Ubiquitin immunohistochemistry demonstrates neurites and neuronal cytoplasmic inclusions (NCI) (arrows) in layer II of the frontal neocortex (**a**), NCI in hippocampal dentate granule cells (**b**), and neuronal intranuclear inclusions (NII) in the superficial neocortex (**c**). **d–f**, *PGRN* immunohistochemistry was positive in some neocortical neurons (**d**), but did not stain NCI or NII in layer II cortex (**e**). Activated microglia (arrows) showed strong *PGRN* expression in affected areas of FTD (**e**) and around senile plaques in Alzheimer's disease (**f**). Scale bars, 30 μ m.

Table 1 | Families with premature termination mutations in PGRN

Family	Origin	Affecteds*	Mean age onset	Mutation (nucleotide)	Mutation (protein)
F161	USA	1 (1)	Early 50s	c.2T>C	p.M1?
UBC17†	Canada	17 (4)	58	c.90_91insCTGC	p.C31LfsX34
1083†	Netherlands	19 (1)	65	c.373C>T	p.Q125X
UBC11	Canada	6 (1)	68	c.388_391delCAGT	p.Q130SfsX124
F53	UK	3 (1)	60	c.388_391delCAGT	p.Q130SfsX124
UBC19	Canada	9 (1)	61	c.IVS8+1G>A	p.V279GfsX4‡
F129	USA	3 (2)	54	c.1145delC	p.T382SfsX29
UBC4	Canada	7 (1)	65	c.1157G>A	p.W386X
UBC15	Canada	10 (4)	60	c.1252C>T	p.R418X
F337	UK	5 (1)	59	c.1402C>T	p.Q468X

*Number of affecteds with NII pathology confirmed is in parentheses.

†Families with previously published linkage to chr17.

‡Predicted effect of IVS8+1G>A mutation.

with our subsequent identification of a *PGRN* mutation that destroys the normal Kozak sequence by altering the Met 1 codon (c.2T>C, M1?) in another tau-negative FTD case (F161) with ub-immunoreactive pathology and NII (Table 1, Fig. 1, Supplementary Fig. 4). This mutation was absent in 200 North American controls and sequence analysis of brain complementary DNA showed a substantial reduction in mutant mRNA, providing evidence of a null allele (Supplementary Fig. 4). A second mutation that alters the Met 1 codon (c.3G>A) was also observed in a Belgian FTD patient series²³.

Next, immunohistochemistry was performed on post-mortem brain tissue using a panel of antibodies that recognize all regions of the PGRN protein. Consistent with previously published data on the distribution of PGRN^{15,16,25}, immunoreactivity was observed in a subset of cortical neurons and intensely in activated microglial cells, both in patients from FTD families with *PGRN* mutations (UBC17, UBC15 and F129) and in normal aged and Alzheimer's disease subjects (Fig. 2d–f). However, the ub-immunoreactive NCI and NII in the FTD cases with mutations showed no staining for PGRN (Fig. 2e). Although this finding indicates that the disease mechanism does not cause the accumulation of PGRN in these lesions, it leaves the identity of the ubiquitinated protein species in NCI and NII unknown.

Although the function of PGRN in neurons has not yet been determined¹⁴, our findings imply that PGRN is essential for neuronal survival and even partial loss of PGRN eventually leads to neurodegeneration. This supports the more general hypothesis that loss of growth factor support can cause neurodegenerative disease^{26,27}. PGRN is expressed in many tissues and mediates its role in development, wound repair and inflammation by activating signalling cascades that control cell-cycle progression and cell motility¹⁴. These include the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-OH kinase (PI(3)K) cascades^{14,28}, both of which regulate crucial functions in neurons. PGRN also stimulates the induction of other growth factors including vascular endothelial growth factor (VEGF)²⁹. It is also of interest, that although partial loss of PGRN apparently results in adult-onset neurodegenerative disease, increased expression of PGRN has consistently been linked to tumorigenesis^{14,28}.

The identification of mutations in *PGRN* fully resolves a ten-year-old conundrum, namely the genetic basis for FTD linked to chromosome 17²⁰, and explains why multiple families linked to this region lack *MAPT* mutations. The fact that *PGRN* is located within 2 Mb of *MAPT* and mutations in both genes independently yield indistinguishable clinical phenotypes is presumably an extraordinary coincidence. Our findings are also highly reminiscent of the recent identification of probable loss-of-function mutations affecting another secreted factor—angiogenin (ANG)—in patients with MND³⁰. Tau-negative FTD and MND share overlapping clinical spectrums and have similar ub-immunoreactive pathology¹⁹. Furthermore, PGRN and ANG are both potent inducers of angiogenesis and are linked with tumorigenesis^{14,30,31}. Although their precise role

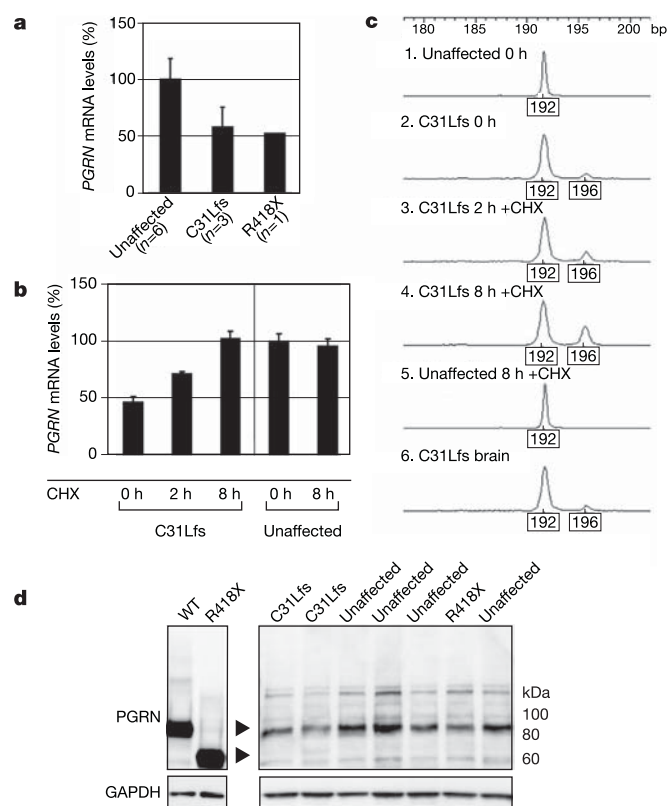


Figure 3 | Mutant *PGRN* mRNAs with premature termination codons are degraded by nonsense-mediated decay. **a**, qRT-PCR analysis shows a ~50% reduction in *PGRN* mRNA in lymphoblastoid cells from individuals carrying the C31LfsX34 (UBC17, $n = 3$) and R418X (UBC15, $n = 1$) mutations. *PGRN* mRNA levels are shown as a percentage of levels in cells from unaffected individuals ($n = 6$). Error bars indicate s.e.m. **b**, Treatment of a C31LfsX34 cell line ($n = 1$) with the NMD inhibitor cycloheximide (CHX; 500 μ M) for 2 h and 8 h results in a progressive increase in total *PGRN* mRNA. *PGRN* mRNA levels are mean values from three replicates. Error bars denote s.d. **c**, RT-PCR fragment analysis in lymphoblastoid cells (trace 2) and brain (trace 6) from patients with the C31LfsX34 mutation shows that the mutant mRNA (196 bp) is virtually absent. Treatment with CHX (traces 3–5) results in the selective increase in C31LfsX34 mutant mRNA. **d**, Western blot analysis of lymphoblastoid extracts shows that wild-type (WT) PGRN protein is reduced in lymphoblasts from mutation carriers (C31LfsX34, $n = 2$ and R418X, $n = 1$) relative to unaffected relatives ($n = 4$) (right panel; mean reduction 34%, $P = 0.01$, t -test). In addition, the C31LfsX34 (UBC17) and R418X (UBC15) mutant proteins are not detected. Arrows indicate wild-type PGRN and the expected position of the R418X mutant protein. R418X PGRN generated from an intronless cDNA construct (not subject to NMD) expressed in HeLa cells (left panel) is included to demonstrate that the mutant protein (if made) is stable.

in neurons has yet to be fully established, it appears that reduced levels of these functionally related factors represents a common mechanism of neurodegeneration in these two diseases. Moreover, these findings imply that replacement of these factors may represent a novel therapeutic strategy in both conditions.

METHODS

See Supplementary Information for further details on the methods used in this study.

PGRN genetic analysis. All *PGRN* exons were amplified from genomic DNA by PCR using the primers listed in Supplementary Table 1, and then sequenced using Applied Biosystems protocol. Following initial identification, the C31LfsX34 mutation was verified with a fluorescent fragment analysis assay, and then checked for both the segregation with disease (in family UBC17) and its presence in 550 North American controls. Sequence analysis of all *PGRN* exons was used to screen 200 aged North American and 95 UK control individuals for all remaining mutations. Segregation of all other mutations in relevant families was checked by sequencing of relevant exons.

Immunohistochemical methods. Immunohistochemistry was performed on post-mortem tissue sections of frontal cortex and hippocampus from two cases of familial FTD with *PGRN* mutations (UBC17 and UBC15), one case of Alzheimer's disease, and one age-matched control individual. The primary antibodies used recognize ubiquitin and all regions of the *PGRN* protein including the amino terminus, carboxy terminus, and the full-length recombinant human *PGRN* protein.

Analysis of *PGRN* protein and *PGRN* RNA. *PGRN* protein levels were quantified by western blot analysis in extracts from lymphoblastoid cells from patients and unaffected relatives using primary antibodies to the N terminus of human *PGRN*, and to GAPDH for normalization. *PGRN* mRNA levels were analysed in patients and unaffected relatives by qRT-PCR using SYBR green on RNA isolated from lymphoblastoid cells and from brain samples. Mass values for *PGRN* mRNA were normalized to 28S ribosomal RNA and divided by *GAPDH* mRNA to determine fold-change in expression. The relative levels of mutant and wild-type *PGRN* mRNA in C31LfsX34 lymphoblasts and brain tissue were analysed by RT-PCR fragment analysis using primers spanning exons 1 and 2. Sequence analysis of RT-PCR products was further used to analyse levels of C31LfsX34 and R418X mutant mRNAs compared with wild-type in brain tissue and lymphoblasts. To study the effect of NMD, lymphoblast cells were treated with cycloheximide for 2–8 h.

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LETTERS

Null mutations in progranulin cause ubiquitin-positive frontotemporal dementia linked to chromosome 17q21

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Frontotemporal dementia (FTD) with ubiquitin-immunoreactive neuronal inclusions (both cytoplasmic and nuclear) of unknown nature has been linked to a chromosome 17q21 region (FTDU-17) containing *MAPT* (microtubule-associated protein tau)^{1–3}. FTDU-17 patients have consistently been shown to lack a tau-immunoreactive pathology^{1–3}, a feature characteristic of FTD with parkinsonism linked to mutations in *MAPT* (FTDP-17)⁴. Furthermore, in FTDU-17 patients, mutations in *MAPT* and genomic rearrangements in the *MAPT* region have been excluded by both genomic sequencing⁵ and fluorescence *in situ* hybridization on mechanically stretched chromosomes⁶. Here we demonstrate that FTDU-17 is caused by mutations in the gene coding for progranulin (*PGRN*), a growth factor involved in multiple physiological and pathological processes including tumorigenesis⁷. Besides the production of truncated *PGRN* proteins due to premature stop codons⁸, we identified a mutation within the splice donor site of intron 0 (IVS0+5G>C), indicating loss of the mutant transcript by nuclear degradation. The finding was made within an extensively documented Belgian FTDU-17 founder family³. Transcript and protein analyses confirmed the absence of the mutant allele and a reduction in the expression of *PGRN*. We also identified a mutation (c.3G>A) in the Met1 translation initiation codon, indicating loss of *PGRN* due to lack of translation of the mutant allele. Our data provide evidence that *PGRN* haploinsufficiency leads to neurodegeneration because of reduced *PGRN*-mediated neuronal survival. Furthermore, in a Belgian series of familial FTD patients, *PGRN* mutations were 3.5 times more frequent than mutations in *MAPT*, underscoring a principal involvement of *PGRN* in FTD pathogenesis.

FTD is the second most common form of dementia in individuals <65 yr of age and manifests clinically with progressive behavioural and personality disturbances including disinhibition, perseveration and emotional blunting, evolving eventually into generalized cognitive impairment⁹. FTD results from severe neuronal degeneration of frontal and/or temporal brain regions. FTDU is the most common pathological subtype of FTD and is characterized by the presence of ubiquitin-immunoreactive neuronal inclusions in the absence of tau pathology¹⁰. In autosomal-dominant FTDU families linkage has been observed with chromosome 9p12–p13 (ref. 11), caused by mutations in

the valosin-containing protein VCP^{11,12}, and 17q21 in the *MAPT* region¹³. Several FTDU families have been conclusively linked to 17q21 (ref. 14) (Fig. 1a), but the underlying gene remained unknown.

We reduced the minimal candidate region of FTDU-17 to 6.3 megabases (Mb) in one Dutch family, 1083 (Fig. 1a), and excluded simple mutations in *MAPT* by exon-based and genomic sequencing^{1,5}. We also excluded the possibility that FTDU-17 resulted from a genomic rearrangement in the *MAPT* locus through using fluorescence *in situ* hybridization (FISH) on mechanically stretched chromosomes⁶—normal patterns were observed in patients of Dutch family 1083 and a recently identified Belgian FTDU-17 founder family³ (Supplementary Fig. 1). Also, comparative genomic hybridization on oligo-based arrays confirmed the absence of a deletion or duplication in the *MAPT* region in both families (data not shown). In addition, biochemical studies of frozen brain tissue had shown normal *MAPT* messenger RNA and protein isoforms^{2,13}. Together, these data supported the notion that FTDU-17 had to be a separate entity in which the ubiquitin-immunoreactive brain pathology was unrelated to tau.

Extensive mutation analyses of other genes from within the 17q21 candidate region identified nonsense and frameshift mutations in the progranulin gene (*PGRN*)⁸. In family 1083, a nonsense mutation Gln125X (exon 4, c.373C>T) was identified in patient III-26, and was absent from 190 control individuals (Supplementary Fig. 2). The mutation segregated with disease (Supplementary Fig. 2a), and nonsense-mediated mRNA decay produced a null allele (Supplementary Fig. 2c). In patients of the Belgian FTDU-17 founder family DR8 (Fig. 2), we identified a G-to-C transversion in intron 0 of *PGRN* at position +5 relative to the first non-coding exon 0 (IVS0+5G>C; Table 1, where IVS indicates intervening sequence), which segregated with disease (Fig. 2). We identified the Belgian founder family based on conclusive 17q21 linkage in one family (DR8 in Fig. 2, LOD score 3.49 at D17S931 (ref. 3)) and subsequent disease haplotype sharing in seven apparently unrelated familial FTD patients (Supplementary Fig. 2b), indicative of a distant common ancestor³. Mutation analysis of *PGRN* in 103 Belgian FTD patients identified the IVS0+5G>C mutation only in the eight probands belonging to the different branches of the Belgian founder family and not in 436 control individuals. In both families, 1083 and DR8, the pathology showed

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the characteristic ubiquitin-immunoreactive neuronal cytoplasmic and nuclear inclusions in the temporal and frontal cortices^{1,3} (Supplementary Table 1 and Supplementary Fig. 3). Rarely, inclusions also involved glia cells, but none of the inclusions was shown to have an appreciable presence for any protein from almost 30 possible proteins tested¹⁵. Also, patients of both families met the clinical criteria of FTD^{10,16}, without associated signs of motor neuron disease^{1,3}.

The IVS0+5G>C mutation is located in the splice donor site of the first *PGRN* intron (intron 0) following the non-coding exon 0 (Table 1 and Fig. 1); *in silico* analysis predicted a marked drop in binding efficiency of the U1 snRNP complex. Furthermore, analysis of full-length *PGRN* complementary DNA in lymphoblasts and brain of probands DR8 (III-28, Fig. 2) and DR27 (III-4, Supplementary Fig. 2b) did not identify aberrant transcripts (Fig. 3a). However, DR8 III-28 and DR27 III-4—who were heterozygous C/T for single-nucleotide polymorphism (SNP) rs5848 located in the 3' untranslated sequence (UTS), with the C allele segregating on the disease haplotype (Fig. 2 and Supplementary Fig. 2b)—showed only the T allele when we sequenced their lymphoblast and/or brain *PGRN* cDNA (Fig. 3b). In contrast, an unaffected relative was heterozygous for rs5848 in both genomic and cDNA sequences. These data support a complete absence of mutant *PGRN* mRNA transcript, most likely due to read-through of intron 0. Nuclear retention signals remaining on the unspliced transcript will prevent it from leaving the nucleus, marking it for nuclear degradation¹⁷. Western blot analysis of lymphoblast cell protein extracts from probands DR8 III-28 and DR27 III-4 supported these data and demonstrated a reduction of *PGRN* protein levels (Fig. 3c). Together, these data demonstrate that the DR8 founder haplotype carries a *PGRN* null allele that does not produce protein. Robust *PGRN* immunoreactivity was observed in a subset of cortical neurons in patient DR8 III-28 (Supplementary Fig. 3a). However, despite using a panel of antibodies that recognize all regions of the *PGRN* protein, the neuronal inclusions were negative for *PGRN*.

In addition to the IVS0+5G>C mutation in the eight probands of the Belgian FTDU-17 founder family, we identified three other *PGRN* mutations in three familial patients of the Belgian FTD series by genomic sequencing of all 13 exons and flanking intronic regions of *PGRN* (Table 1 and Fig. 1b). In one FTD patient, with an onset age of 62 yr and a sister suffering from dementia who died at 64, we identified in exon 1 a G>A transition that destroyed the native Kozak sequence surrounding the Met1 translation initiation codon (c.3G>A). We could not verify the effect of the Met1 mutation on transcript or protein expression because we had no RNA source available for this FTD patient who died without autopsy at age 72 yr. However, supportive data were obtained for a US patient who carried a different Met1 mutation (c.2T>C) showing a substantial reduction in expression levels of the mutant transcript allele⁸. In the two other familial patients, different frameshift mutations predicted carboxy-terminal-truncated proteins, Pro127ArgfsX2 and Ala237TrpfsX4, resulting from a dinucleotide deletion in exon 4 and a four-nucleotide insertion affecting the intron 7 splice donor site and predicting exon 7 skipping (Table 1 and Fig. 1b). Both patients are still alive but no lymphoblasts were available for *PGRN* cDNA or protein verification. However, in a separate study it was shown that *PGRN* frameshift mutations also produce null alleles through nonsense-mediated decay of mutant mRNA transcripts, lowering *PGRN* protein levels⁸. Together, our *PGRN* mutation data explained 10.7% (11 out of 103) of the genetic aetiology of FTD and 25.6% (11 out of 43) of familial FTD in the Belgian patient series. In the same group, *MAPT* mutation frequencies were 2.9% (3 out of 103) overall and 7% (3 out of 43) in familial FTD, indicating that *PGRN* mutations are an approximately 3.5 times more frequent cause of FTD in Belgian patients. The contribution of *PGRN* mutations to the genetic aetiology of FTD might be higher because the methods that we used would not have detected whole-gene deletions or out-frame exonic deletions/duplications resulting in loss of functional *PGRN*, neither did we systematically examine the *PGRN* 5' regulatory region for mutations affecting *PGRN* transcriptional activity. In the Belgian

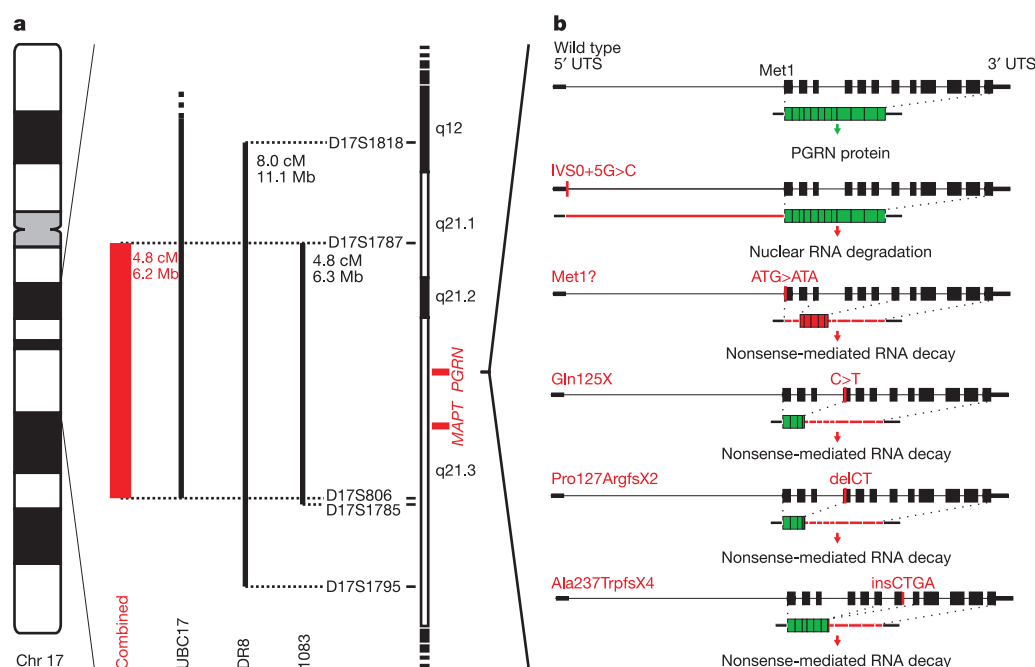


Figure 1 | FTDU-17 candidate region and *PGRN* mutations. **a**, Location of *PGRN* in the FTDU-17 candidate region of chromosome 17q21. The red bar represents the minimal linked region based on a centromeric recombinant in family 1083 (ref. 1) and a telomeric recombinant in family UBC17 (ref. 2). Genetic sizes are according to the Marshfield gender-averaged linkage map; physical sizes according to human reference sequence NCBI build 35.

b, Schematic presentation of *PGRN* mutations in Dutch FTDU-17 family 1083, Belgian FTDU-17 founder family DR8, and in unrelated Belgian FTD patients (Table 1). The predicted changes are shown for the largest *PGRN* transcript (GenBank accession number NM_002087.2). In the transcripts, regions encoding normal protein sequence are coloured green, whereas mutant regions are red.

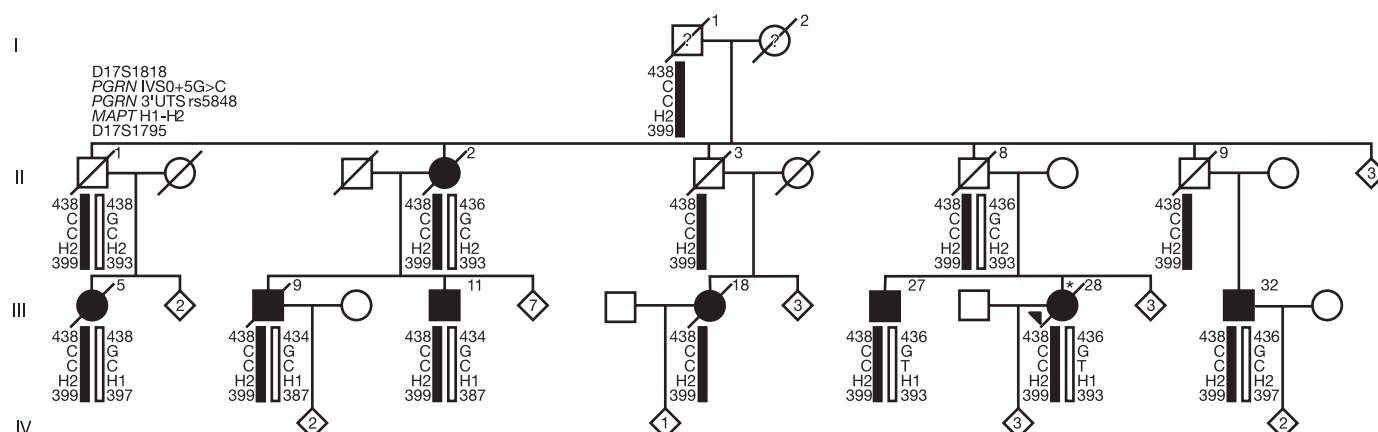


Figure 2 | Segregation of the *PGRN* splice donor site mutation IVS0+5G>C in Belgian founder family DR8. Segregation in the 17q21-linked branch DR8 is shown for microsatellite markers flanking the founder haplotype (Fig. 1), the *PGRN* IVS0+5G>C mutation, the *PGRN* 3' UTS SNP rs5848 used in cDNA analyses (Fig. 3b), and the *MAPT* H1-H2 inversion haplotype analysed in the chromosomal fluorescence *in situ* hybridization (FISH; Supplementary Fig. 1e, f). Black bars represent the disease haplotype of

patients and obligate carriers. In generation I of DR8, the disease haplotype was arbitrarily assigned to individual I-1. Numbers within each diamond are unaffected at-risk individuals that were included in the genotyping. The arrowhead identifies the proband; the asterisk indicates that a pathological diagnosis of FTDU was available for proband III-28 (Supplementary Table 2 and Supplementary Fig. 3a). Filled symbols represent patients; open symbols unaffected relatives.

founder family the 16 patients carrying the IVS0+5G>C mutation had onset ages varying between 45 and 70 yr (mean onset age 63.4 ± 6.8 yr; mean age at death 68.3 ± 4.4 yr). There were also four obligate carriers in generation II of family DR8 (Fig. 2) who died without symptoms of dementia: one died at a young age (II-1, 41 yr), two at ages within the onset range (II-8 at 44 yr and II-9 at 54 yr) and one at 81 yr (II-3)³. These highly variable onset ages and potential incomplete penetrance of the disease indicated that modifying factors are modulating onset age and as such contribute to a more complex genetic aetiology for FTDU-17. A first analysis of the apolipoprotein E gene (*APOE*) indicated that the *APOE* genotype has no effect on onset age (data not shown). Also of interest is that many of the FTDU-17 patients in the Belgian founder family had symptoms of non-fluent aphasia as a prominent feature of their disease (Supplementary Table 1)³.

Our mutation data for *PGRN* explained linkage of FTDU-17 in Dutch family 1083 (ref. 1) and the Belgian founder family DR8 (ref. 3). Although studies of nonsense and frameshift mutant transcripts indicated that they were probably degraded by nonsense-mediated mRNA decay (Supplementary Fig. 2c)⁸, it could not be fully excluded that undetected low amounts of truncated proteins exerted their pathogenic effect through a dominant-negative or gain-of-function

mechanism. However, identification of a loss-of-allele mutation in intron 0, IVS0+5G>C, provided convincing evidence that the pathogenic mechanism in FTDU-17 is indeed a loss of functional *PGRN* (haploinsufficiency). The IVS0+5G>C mutation either prevents splicing out of the first intron, intron 0, causing nuclear retention and degradation of the mutant transcript, or the mutant allele is never transcribed. Either way, the mutant allele is non-functional and the final result is a reduction in *PGRN* protein.

PGRN is a member of a family of cysteine-rich polypeptides with growth modulatory activity and a role in several physiological and pathological processes. In brain, *PGRN* is widely expressed in neurons and glia cells but its actual functions are not very well understood¹⁸. *PGRN* has been implicated in the development of male-specific differentiation of the hypothalamus, and it is highly expressed in glioblastomas^{19,20}. Loss of functional *PGRN* in FTDU-17 and potentially also in other neurodegenerative brain diseases^{21,22} supports a role for *PGRN* in neuronal survival. However, although partial deficiency of *PGRN* causes FTDU-17, overexpression of *PGRN* has been linked to the progression of many different cancers, indicating that *PGRN* dosage is important in these diseases. Nevertheless, the identification of a role for *PGRN* in neurodegeneration opens new avenues for treatment, as *PGRN* has been shown to

Table 1 | *PGRN* mutations identified in Belgian FTD patients

Patient/family	Pathology†	Onset age in years	Genome‡	Mutation* Predicted RNAs	Predicted protein	Location
DR8	FTDU	63.8 (n = 5)	g.96241G>C (IVS0+5G>C)	-	p.0	IVS 0
DR2	Alive	66.3 (n = 4)	g.96241G>C (IVS0+5G>C)	-	p.0	IVS 0
DR25	FTDU	69.5 (n = 2)	g.96241G>C (IVS0+5G>C)	-	p.0	IVS 0
DR26	Alive	65	g.96241G>C (IVS0+5G>C)	-	p.0	IVS 0
DR27	FTDU	58	g.96241G>C (IVS0+5G>C)	-	p.0	IVS 0
DR28	FTDU	57	g.96241G>C (IVS0+5G>C)	-	p.0	IVS 0
DR31	FTDU	66	g.96241G>C (IVS0+5G>C)	-	p.0	IVS 0
DR119	Alive	45	g.96241G>C (IVS0+5G>C)	-	p.0	IVS 0
DR118	Died without autopsy	62	g.100069G>A	c.3G>A	p.Met1?¶	EX 1
DR120	Alive	56	g.101160_101161delCT	c.380_381delCT	p.Pro127ArgfsX2	EX 4
DR91	Alive	67	g.102065_102066insCTGA	c.709_835del	p.Ala237TrpfsX4	IVS 7

*Positions of the different *PGRN* mutations are depicted in Fig. 1. Sequencing all 13 exons of *PGRN* in 190 control individuals did not identify these or other nonsense or frameshift mutations. Sequencing of exon 0 in 246 additional control individuals showed that the IVS0+5G>C mutation was absent.

†FTDU is an autopsy brain pathology diagnosis.

‡Numbering relative to the reverse complement of GenBank accession number AC003043 and starting at nucleotide 1.

§Numbering according to the largest *PGRN* transcript (GenBank accession number NM_002087.2) and starting at translation initiation codon.

||Numbering according to the largest *PGRN* isoform (GenPept accession number NP_002078.1).

¶Mutation in Met1 translation initiation codon.

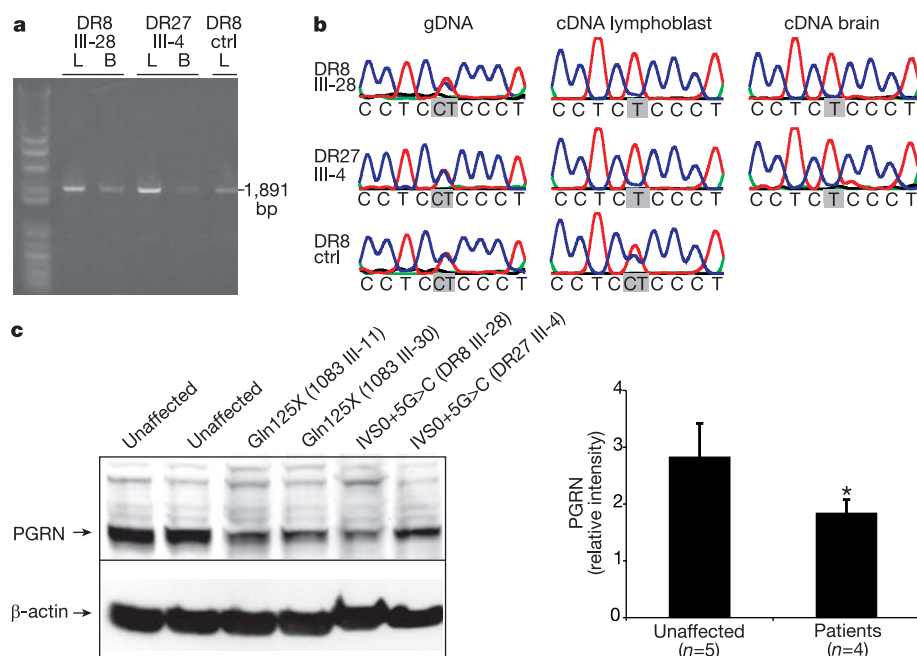


Figure 3 | Transcript and protein analyses of the *PGRN* IVS0+5G>C mutation. **a**, Whole-length *PGRN* PCR amplicons obtained from first-strand cDNA prepared from lymphoblast (L) or brain (B) of probands DR8 III-28 and DR27 III-4 (Fig. 2 and Supplementary Fig. 2b), and an unaffected relative in DR8 showing the expected transcript length of 1,891 bp. **b**, Sequence of SNP rs5848 located in the 3' UTS of *PGRN* in gDNA and cDNA prepared from lymphoblasts or brain of probands DR8 III-28 and DR27 III-4, and an unaffected relative in DR8 heterozygous for SNP rs5848. Amplicons were prepared from first-strand cDNA using PCR primers in

exon 5–6 and 3' UTS amplifying all known *PGRN* transcripts. **c**, Analysis of wild-type *PGRN* protein normalized to β -actin from lymphoblasts of patients DR8 III-28 (Fig. 2), DR27 III-4 (Supplementary Fig. 2b) and unaffected relatives, and of patients of Dutch family 1083 segregating the Gln125X mutation (Supplementary Fig. 2a). Normalized *PGRN* staining in lymphoblasts of IVS0+5G>C and Gln125X carriers was reduced by 30–35% compared to unaffected relatives. Arrows indicate the wild-type *PGRN* (~85 kDa) and β -actin. Error bars represent s.e.m.; asterisk, $P = 0.028$ (t -test).

stimulate other growth factors such as vascular endothelial growth factor (VEGF)²³ associated with adult-onset progressive motor neuron degeneration in mice²⁴.

METHODS

Patients. Belgian patients had pure FTD and were diagnosed using a standard protocol and established clinical criteria^{9,25}. In the series of 103 patients, ten patients had a definite diagnosis of FTDU, two of dementia lacking distinctive histopathology (DLHD) and one of Pick's disease. Previous mutation analyses identified a *MAPT* mutation in three patients, Gly273Arg, Ser305Ser and Arg406Trp, and one presenilin 1 (*PSEN1*) mutation, Gly183Val, in the patient with Pick's pathology²⁶. In the whole sample, mean onset age was 63.8 ± 9.1 yr (range 40–90 yr); there were 50 females and 53 males and 43 patients had a positive family history with at least one first-degree relative affected. The FTD series included eight probands sharing the same haplotype at 17q21, indicative of a common founder³. The local medical ethical committee of the University of Antwerp approved the research protocols for clinical, genetic and neuropathological studies.

***PGRN* gene sequencing.** The sequence of non-coding exon 0 and coding exons 1–12 was determined in the 103 Belgian FTD patients and 190 neurologically healthy control individuals (mean age 52.4 ± 13.3 yr, range 37–85 yr). For the *PGRN* exon 0 fragment containing the IVS0+5G>C mutation, 246 additional control individuals (mean age 67.0 ± 12.8 yr, range 40–92 yr) were sequenced. Total genomic DNA was prepared from peripheral blood according to standard procedures. Standard 20- μ l polymerase chain reaction (PCR) amplifications on genomic DNA were performed to amplify exons including exon–intron boundaries with primers designed using Primer 3 (ref. 27) (Supplementary Table 2). Amplification products were purified with 1 U Antarctic phosphatase (New England Biolabs) and 1 U exonuclease I (New England Biolabs) and sequenced in both directions using the BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems) on an ABI3730 automated sequencer (Applied Biosystems). Sequences were analysed with the Software Package NovoSNP²⁸.

***PGRN* mRNA and protein analyses.** Epstein–Barr virus (EBV)-transformed lymphoblasts were cultured and mRNA was isolated using the Chemagic mRNA Direct Kit (Chemagen). Frontal brain tissue from the patients was homogenized

and total RNA was extracted using the RiboPure Kit (Ambion). First-strand cDNA was synthesized starting from mRNA or total RNA with random hexamer primers using the SuperScript III First-Strand Synthesis System for RT–PCR kit (Invitrogen). PCR was performed on both lymphoblast and brain cDNA using primers amplifying the complete coding region of the *PGRN* transcript and primers amplifying part of the transcript containing exon 5–6 to 3' UTS. Primer sequences are summarized in Supplementary Table 2. The resulting PCR products were sequenced to detect aberrant transcripts and to determine the number of transcribed alleles based on the presence of SNP rs5848.

Lymphoblasts were collected by centrifugation at 250 g and lysed in homogenization buffer. Samples were sonicated, cleared at 20,000 g and protein aliquots (40 μ g) separated on a 4–12% Bis-Tris Nupage gel (Invitrogen) and were electroblotted to Hybond P polyvinylidene difluoride membrane (Amersham Biosciences). Membranes were immunoblotted with anti-*PGRN* antibodies (acroganin N-19 and S-15) and detected with secondary antibody and ECL plus chemiluminescent detection system (Amersham Biosciences) with bands quantified on Kodak Imaging Station 440 (Eastman Kodak). Quantitative data were normalized to the signal obtained for β -actin (clone AC-15; Sigma).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Highly ordered arrangement of single neurons in orientation pinwheels

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In the visual cortex of higher mammals, neurons are arranged across the cortical surface in an orderly map of preferred stimulus orientations^{1,2}. This map contains ‘orientation pinwheels’, structures that are arranged like the spokes of a wheel such that orientation changes continuously around a centre. Conventional optical imaging^{3,4} first demonstrated these pinwheels^{3,5}, but the technique lacked the spatial resolution to determine the response properties and arrangement of cells near pinwheel centres. Electrophysiological recordings later demonstrated sharply selective neurons near pinwheel centres^{6,7}, but it remained unclear whether they were arranged randomly or in an orderly fashion. Here we use two-photon calcium imaging *in vivo*^{8–12} to determine the microstructure of pinwheel centres in cat visual cortex with single-cell resolution. We find that pinwheel centres are highly ordered: neurons selective to different orientations are clearly segregated even in the very centre. Thus, pinwheel centres truly represent singularities in the cortical map. This highly ordered arrangement at the level of single cells suggests great precision in the development of cortical circuits underlying orientation selectivity.

A smooth map for orientation preference in the mammalian visual cortex was first demonstrated by Hubel and Wiesel^{1,2}, who recorded from neurons along electrode penetrations parallel to the cortical surface. They found occasional abrupt discontinuities in the sequence of preferred orientations, but point-by-point sampling with microelectrodes did not permit an overall characterization of the orientation map. Only several decades later did optical imaging of intrinsic signals reveal that the orientation preference map is composed of pinwheel-like structures^{3,5}. Primarily as a result of light scatter in cortical tissues, conventional optical imaging^{3,4} has a lateral spatial resolution of 50 μm at best¹³, and very limited depth resolution, so it is not capable of resolving the fine-scale structure of the pinwheel centres. Similarly, previous electrophysiological studies of pinwheel centres⁶ could not localize neurons precisely (for instance, tetrodes record in a region with a radius of about 65 μm (ref. 14)). Thus, below this spatial scale, they could not resolve whether neurons selective for different orientations were arranged randomly near the pinwheel centre or might be perfectly segregated (see Supplementary Fig. S1). Two-photon calcium imaging^{8–12} permits functional mapping at single-cell resolution¹⁰ in three dimensions, so it is ideally suited to resolve this question.

To target the injection of the calcium indicator dye to a pinwheel centre, we first recorded maps of preferred orientation in area 18 of cat visual cortex (postnatal days 28–35) with intrinsic-signal optical imaging (Fig. 1a). An injection of Oregon Green BAPTA-1 acetoxymethyl ester (AM) at a pinwheel site typically labelled thousands of neurons in a region with a diameter of 300–600 μm . Two-photon calcium imaging permitted the simultaneous measurement of the

visual responses of tens to hundreds of labelled neurons within a given optical cross-section parallel to the cortical surface.

When we measured calcium signals evoked by visual stimuli (gratings at four different orientations, each drifting in two opposite directions), we found that neurons responsive to each orientation and direction were tightly clustered around a pinwheel (Fig. 1b). In cell-based orientation maps (see Methods), we plotted the preferred orientation of each neuron and found that orientation varied smoothly over the cortical surface with very little scatter (Fig. 1c–e). In the experiment illustrated in Fig. 1b, c, data were collected at nine depths spanning 160 μm . When cells from all depths were superimposed (Fig. 1c, lower right; see also Supplementary Video 1), the orientation maps matched precisely and the pinwheel centre was immediately evident. This demonstrates the columnar structure of the orientation map at a very fine spatial scale. Even near the pinwheel centre there was virtually no mixing of cells with different orientation preferences.

The orientation tuning of individual neurons is best appreciated by examining the time course of the calcium signals evoked by visual stimulation (see also Supplementary Video 2 for a time-lapse movie of the calcium signal changes). Figure 2 shows three examples of individual neurons close to a pinwheel centre (less than 65 μm , Fig. 2b) and in an iso-orientation domain at some distance from the centre (more than 65 μm , Fig. 2c). The radius of 65 μm (Fig. 2a) was chosen to approximate the recording sphere of the multi-electrodes (tetrodes) used in earlier studies¹⁴. As shown previously (Fig. 2 in ref. 6), the preferred orientations of the cells close to the pinwheel centre were frequently very different from each other (Fig. 2d); for instance, cells 1 and 3 (Fig. 2b) were tuned to orthogonal orientations. In contrast, cells in a neighbourhood of the same diameter in the iso-orientation domain (Fig. 2e) were all tuned to nearly identical orientations.

We examined 6,616 cells from ten pinwheels in nine animals (2–17 depths were imaged for each pinwheel). In all, 1,002 cells were located in what we call the pinwheel centre (less than 65 μm from the singularity; see Supplementary Methods). Of these, 796 (79.4%) were selective for orientation, 41 (4.1%) were non-selective but responsive, and 165 (16.5%) were unresponsive. In the periphery (more than 65 μm from the centre), out of 5,614 cells, 5,230 (93.2%) were selective, 59 (1.1%) were non-selective but responsive, and 325 (5.8%) were unresponsive.

To demonstrate that the preferred orientations of cells changed smoothly and progressively around the pinwheel, the preferred orientations of all selective cells for each pinwheel were plotted against the azimuth (θ in Fig. 3a), the angular positions of cells with respect to the pinwheel centre (Fig. 3b). As illustrated in Fig. 1c, the relation between the cells’ preferred orientation and angular positions was well fitted by a smooth curve (black curve in Fig. 3b, the

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azimuth–orientation function) derived from cells located in the periphery of the pinwheel (more than $65\ \mu\text{m}$ from the centre; blue points). Cells in the pinwheel centre (less than $65\ \mu\text{m}$; red points) follow the same curve (correlation coefficient $r = 0.98$). Thus, cells in the centre of the pinwheel are arranged according to the pattern seen in the periphery.

We further examined the relationship between pinwheel centre and periphery by quantifying three parameters: the orderliness of the orientation map, the response strength, and the orientation tuning width. First, we measured the angular deviation, which is the degree to which individual cells deviated from the orderly pinwheel arrangement, expressed in the azimuth–orientation function (black curve in Fig. 3b) for each pinwheel. For cells close to the pinwheel centre, the distribution of angular deviations was clearly biased towards zero (Fig. 3c; median 9°), although they were somewhat higher than in the surround (Fig. 3d; median 5° (see also Supplementary Fig. S2); however, if the deviation was measured as cortical displacement of neurons, it was smaller at the centre (see Supplementary Fig. S3)). The distribution in the centre was significantly different from the random distribution obtained by shuffling the location of cells (Fig. 3c, grey bars; $P < 10^{-10}$; Wilcoxon rank-sum test). Second, the response amplitudes in the pinwheel centre (Fig. 3f; median 4.3% fluorescence increase) were smaller than in the periphery (Fig. 3g; 5.8%, $P < 10^{-12}$; Wilcoxon rank-sum test). Finally, cells close to the pinwheel centre were selective to orientation but had a slightly broader tuning bandwidth (Fig. 3i; median 37°) than cells in the periphery (Fig. 3j; median 31° ; $P < 10^{-4}$; Wilcoxon rank-sum test). The differences in tuning width were sufficiently robust that they were observed independently in different subsets of the data (even and odd trials, Supplementary Figs S4 and S5).

We found essentially the same relationship between the pinwheel centre and periphery in all ten pinwheels studied. The pinwheel

centres were remarkably well organized: the median angular deviation of the measured preferred orientation from the azimuth–orientation function was small (less than 17°), although consistently larger than in the periphery (Fig. 3e). The median response strength of the cells was always 17–41% smaller in the pinwheel centre than in the periphery (Fig. 3h). The median bandwidth of orientation tuning was consistently broader in the pinwheel centre than in the periphery, but this difference was always small (less than 11° ; Fig. 3k).

The present study was performed with kittens at an age when orientation maps are well established (postnatal days 28–35)^{15,16}, but still within the critical period. Our results are consistent with the idea that tuning width might be slightly broader in the pinwheel centres of kittens of this age, as has been suggested¹⁷. However, any quantitative conclusions about the degree of orientation tuning must take several technical issues into consideration. First, to minimize experiment time, we sampled orientation coarsely (45°) in most experiments. When we sampled orientation more densely (22.5°) in some experiments, the apparent tuning width of the most selective cells became considerably smaller (see Supplementary Figs S6 and S7), which rather enlarged the difference in tuning width between centre and periphery. Second, the smaller responses at the pinwheel centres might result in apparently broader curves due to decreased signal-to-noise ratios; indeed, the measured tuning width was inversely correlated with response strength both near the centre and in the periphery (Supplementary Fig. S8).

The smaller responses near the pinwheel centres than in the periphery might also have contributed to the apparently higher percentage of unresponsive cells in the centre (16.5%) than in the periphery (5.8%). Alternatively, the unresponsive cells at the pinwheel centres might have been selective to some other stimulus attributes. In a few experiments we tried square-wave gratings at a range of spatial frequencies (0.07–1.0 cycles/degree) at a single

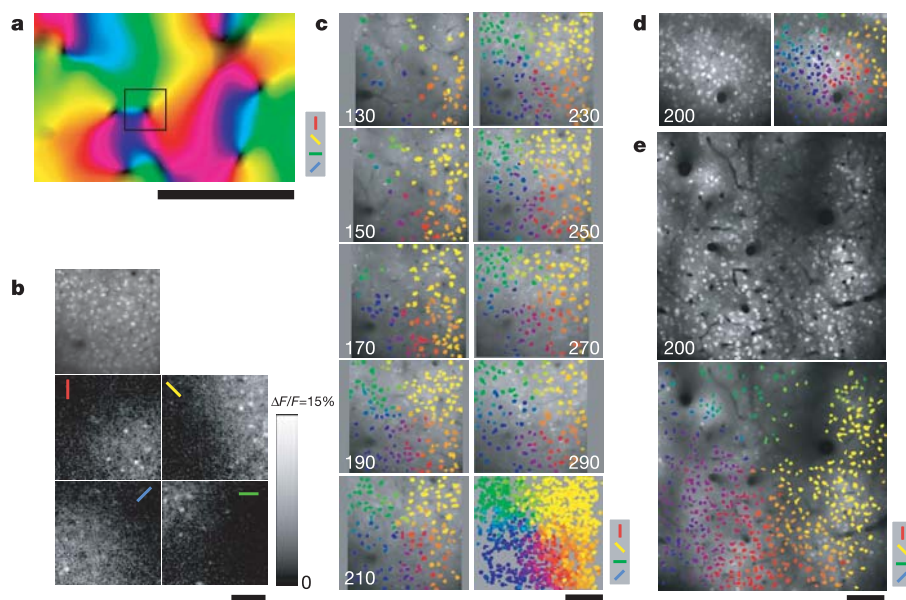


Figure 1 | Functional maps of orientation pinwheels. Pinwheels were mapped at low resolution (**a**) and with single-cell resolution (**b–e**). **a**, An orientation map obtained with intrinsic-signal optical imaging. In this colour-coded map (polar map), hue is determined by the best orientation. Darker colours, in pinwheel centres, represent less selective responses. **b**, Two-photon calcium imaging. Approximately, the square region drawn in **a** was imaged at $250\ \mu\text{m}$ below the pial surface. The top panel shows an averaged image of cortical cells stained with the calcium indicator Oregon Green 488 BAPTA-1 AM. The bottom four panels show single-condition maps for four orientations of visual stimuli ($\Delta F/F$, the percentage change in fluorescence between stimulation period and blank; gaussian smoothed

by $1\ \mu\text{m}$). The scale bar ($\Delta F/F$) applies only to the bottom four panels. **c**, Cell-based orientation maps from nine different depths (130, 150, 170, 190, 210, 230, 250, 270 and $290\ \mu\text{m}$, as indicated). Selective cells (1,034 out of 1,055 cells; $P < 0.05$, ANOVA across eight directions) are coloured according to their preferred orientation. The cortical surface was tilted (about 15°), which was corrected for by shifting the images by $5.2\ \mu\text{m}$ for every $20\ \mu\text{m}$ in depth (grey margins indicate this shift). The last panel shows the overlay of images from all nine depths. **d**, **e**, Dye-loaded cells and orientation maps in pinwheels from two other animals. Scale bars, 1 mm (**a**); $100\ \mu\text{m}$ (**b–e**).

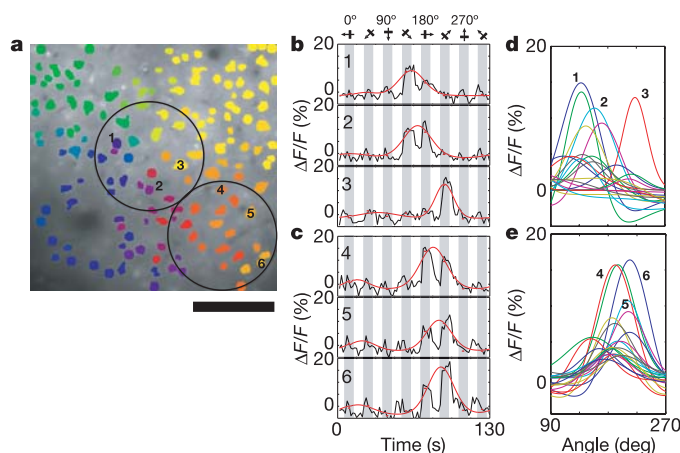


Figure 2 | Tuning curves of cells close to a pinwheel centre and in an iso-orientation domain. **a**, A cell-based orientation map from 230 μm below the pial surface (redrawn from Fig. 1c). Cells were sampled from two circular regions (radius 65 μm), one at the pinwheel centre and the other in an iso-orientation domain. Scale bar, 100 μm . **b**, **c**, Time courses of three selective cells (1–3) in the pinwheel centre (**b**) and three selective cells (4–6) in the iso-orientation domain (**c**). Grey bars indicate periods of visual stimulation—oriented gratings drifted in eight directions—which were interspersed with blank stimuli of uniform luminance. Black traces show the average response to ten repeats; red traces show the orientation tuning curves fitted to the peak responses. **d**, **e**, Tuning curves of all the cells in the pinwheel centre (**d**) and the iso-orientation domain (**e**). Tuning curves of cells 1–6 are numbered.

orientation, but observed similar results. We did not examine whether more complex stimuli, such as corners or other contextual influences¹⁸, might preferentially excite neurons in pinwheel centres.

Previously we reported the existence of precise borders¹⁰—direction fractures—between cells with opposite direction preferences in cat area 18. In theory, direction fractures must intersect with pinwheel

centres because one cycle of orientation (180°) cannot span a full cycle of directions (360°; see refs 19–21). In seven out of ten pinwheels we examined, a well-defined direction fracture intersected the pinwheel centre (Supplementary Fig. S9). In the other three cases, the arrangement was less clear.

Two-photon calcium imaging is unique in its ability to determine not only the physiological response of hundreds of cells simultaneously but also their precise location in the cortical circuit. This enabled us to explore the functional micro-architecture of orientation pinwheels in cat visual cortex and to prove that pinwheel centres truly represent singularities in the cortical orientation map. These singularities are substantially different from a linear border, such as a direction fracture^{10,20,21}. With a linear border, if a neuron's dendritic arbour is symmetric, more than half will overlap cells with similar properties. Near a pinwheel centre, however, any symmetric dendritic arbour would overlap predominantly with regions of different orientation preferences.

Several different mechanisms might be responsible for the sharp orientation tuning of neurons near the pinwheel centre, despite the fact that most of their nearest neighbours have different orientation preferences. First, the cell bodies in the centre of pinwheels might have dendrites that are strongly biased towards the nearby domain with the same orientation (Supplementary Fig. S10a). Alternatively, the overall dendritic arbour might be symmetric, with little or no bias, but there could be stronger or more numerous synapses along dendrites in the iso-orientation domain (Supplementary Fig. S10b). Even with symmetric inputs around the dendritic tree, however, sharp tuning could still be achieved. If synapses proximal to cell bodies were stronger than distal synapses (Supplementary Fig. S10c), then neurons only slightly displaced from the centre would be sharply tuned, provided that local afferent inputs are themselves tightly segregated. Finally, even if symmetric neurons integrated inputs over a large dendritic area (Supplementary Fig. S10d), weakly biased orientation tuning could be sharpened by a nonlinear input–output transformation, such as the threshold for spike generation^{7,22}.

With any one of these mechanisms of sharp orientation tuning, the

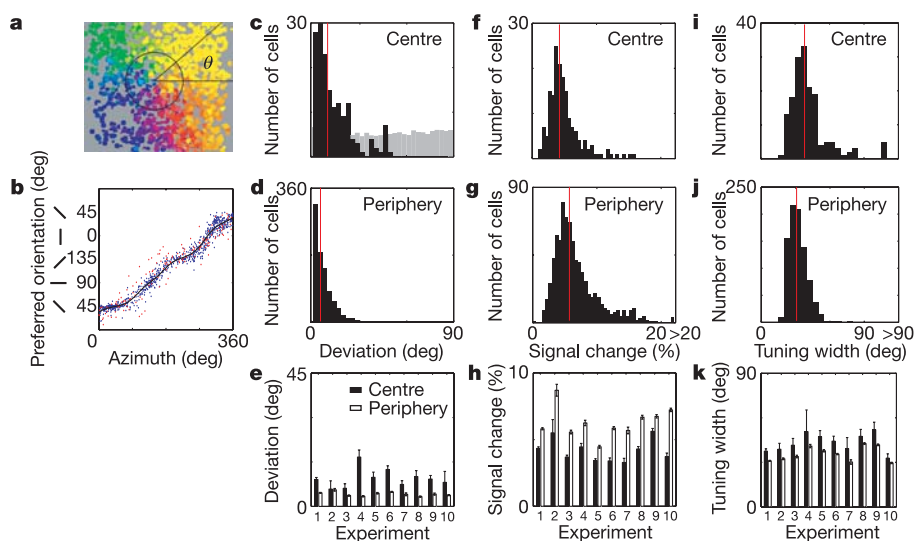


Figure 3 | Relationship between neurons in the pinwheel centre (less than 65 μm from singularity) and in the periphery (more than 65 μm). **a**, An overlay of cell-based orientation maps from nine depths (redrawn from Fig. 1c). **b**, Relation between cell positions (azimuth; defined as θ in **a**) and the preferred orientations. Red dots represent cells in the pinwheel centre, blue dots cells in the periphery. All selective cells (1,034 out of 1,055 cells from nine depths) are included. The azimuth–orientation function (black trace) was obtained by fitting the blue dots with a smooth curve. **c**, **d**, Histograms of angular deviation of preferred orientation from the average (black trace) in **b** for all the selective cells near the pinwheel centre

(**c**) and in the periphery (**d**). The grey histogram in **c** shows the random distribution obtained by shuffling the location of cells. Red lines, median values (see the text). **e**, Median angular deviation for cells in the pinwheel centre (filled bars) and the periphery (open bars) for all ten pinwheels studied. **f**, **g**, Histograms of change in fluorescence evoked by optimal orientation near the pinwheel centre (**f**) and in the periphery (**g**). **h**, Median signal changes in all pinwheels. **i**, **j**, Histograms of tuning width (half-width at half-maximum) for all the cells near the pinwheel centre (**i**) and in the periphery (**j**). **k**, Median tuning widths in all pinwheels. Where shown, error bars indicate s.e.m.

question would still remain: what is the developmental process that yields the precise arrangement of neurons in a pinwheel centre? It is known that the macroscopic architecture of pinwheels is established by the time of eye opening in the ferret²³ and is stable throughout the critical period in the cat^{15,16}. If neurons received many inputs over large dendritic arbours, then their outputs would represent a smooth interpolation of the macroscopic map. Thus, a pinwheel centre could be spatially precise even if each cell is broadly tuned early in development¹⁷. The sharpening of their orientation tuning could then be achieved by hebbian mechanisms that might, for instance, selectively prune dendritic arbours (Supplementary Fig. S10a) or strengthen specific synaptic connections (Supplementary Fig. S10b). Alternatively, the broadly tuned inputs in the pinwheel centre might be sharpened in the adult by a threshold operation, so there is no need for morphological or synaptic refinement to achieve sharp tuning⁷ (Supplementary Fig. S10d).

The discovery of macroscopic functional architecture initiated studies of the relationship between function and morphology of axonal arbours (see, for example, refs 24, 25) and dendritic trees^{26–29}. The demonstration of precise micro-architecture in the present study suggests a relationship between functional maps and anatomy at an even finer spatial scale. Neuronal operations at this scale will be understood only when microscopic functional imaging is combined with higher-resolution techniques that elucidate neuronal morphology and perhaps even individual synaptic connections.

METHODS

Cats (postnatal days 28–35) were anaesthetized with isoflurane (1–2% in surgery, 0.5–1% during imaging; see Supplementary Methods for details). A small craniotomy was performed over the visual cortex, the dura reflected and the underlying cortex covered with 3% agarose. For intrinsic-signal optical imaging, the cortex was illuminated with 630-nm light and the camera (Imager 3001; Optical Imaging) was focused 400 μm below the cortical surface. Using the surface blood vessel pattern, we targeted a pinwheel centre with a glass pipette to inject the cell-permeant calcium indicator (0.8 mM Oregon Green 488 BAPTA-1 AM with 8% dimethylsulphoxide, 2% pluronic acid and 40 μM Alexa-594; all from Molecular Probes). Changes in calcium fluorescence of cortical neurons were monitored with a Leica TCS SP2 microscope coupled with a Mira 900 (Coherent Systems) mode-locked Ti:sapphire laser (810 nm). For visual stimulation, square-wave gratings were drifted at 2 Hz at 8 or 16 directions of motion in steps of 22.5° or 45°. Each stimulus started with a blank period (8 s) followed by the same period of visual stimulation. The 8 or 16 stimuli were presented sequentially and repeated 10 times. Two-photon images were analysed in Matlab (Mathworks) and ImageJ (National Institutes of Health). Cells were automatically identified (6,616 cells in 10 pinwheels) through a series of morphological filters that delineated the contours of cell bodies. Visually responsive cells were defined by analysis of variance (ANOVA) across the blank and 8 or 16 directions ($P < 0.05$; 6,126 cells, 92.6%). Of these, selective cells were defined by ANOVA across 8 or 16 directions ($P < 0.05$; 6,026 cells, 91.1%). Preferred orientation was obtained by vector averaging. Tuning curves were fitted with the sum of two circular gaussian functions³⁰.

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NMDA-receptor-mediated, cell-specific integration of new neurons in adult dentate gyrus

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New neurons are continuously integrated into existing neural circuits in adult dentate gyrus of the mammalian brain^{1–4}. Accumulating evidence indicates that these new neurons are involved in learning and memory^{5–8}. A substantial fraction of newly born neurons die before they mature^{9,10} and the survival of new neurons is regulated in an experience-dependent manner^{5,6,11}, raising the possibility that the selective survival or death of new neurons has a direct role in a process of learning and memory—such as information storage—through the information-specific construction of new circuits. However, a critical assumption of this hypothesis is that the survival or death decision of new neurons is information-specific. Because neurons receive their information primarily through their input synaptic activity, we investigated whether the survival of new neurons is regulated by input activity in a cell-specific manner. Here we developed a retrovirus-mediated, single-cell gene knockout technique in mice and showed that the survival of new neurons is competitively regulated by their own NMDA-type glutamate receptor during a short, critical period soon after neuronal birth. This finding indicates that the survival of new neurons and the resulting formation of new circuits are regulated in an input-dependent, cell-specific manner. Therefore, the circuits formed by new neurons may represent information associated with input activity within a short time window in the critical period. This information-specific addition of new circuits through selective survival or death of new neurons may be a unique attribute of new neurons that enables them to play a critical role in learning and memory.

The hypothesis that information-specific new circuits are formed through the selective survival/death of new neurons (Fig. 1) is based on an assumption that the survival of new neurons is regulated by input activity in a cell-specific manner. Conventional techniques to block synaptic activity, wherein neuronal activity is globally inhibited, cannot distinguish input-dependent, cell-specific regulation from an activity-dependent mechanism, by which the activity of surrounding neurons would indirectly modulate the survival of new neurons¹² (Supplementary Fig. 1). To test input-dependent, cell-specific regulation would require inhibition of synaptic activity in a small number of new neurons without affecting the activity of neighbouring neurons. For this purpose, we developed a retrovirus-mediated, single-cell knockout technique. We constructed a retroviral vector^{3,13} to express Cre recombinase fused to green fluorescent protein (GFP)¹⁴ under the control of the cytomegalovirus immediate early enhancer-chicken β -actin hybrid (CAG) promoter (CAG–GFP/Cre). We reasoned that injection of this viral vector into the dentate gyrus of adult ‘floxed’ mice, in which the gene of interest is flanked by target sequences of Cre recombinase, would lead to the selective removal of the gene¹⁵ in newborn neurons of adult mice (Fig. 2a). In this study, we decided to focus on a component of synaptic activity mediated by an *N*-methyl-D-aspartate-type

glutamate receptor (NMDAR). This receptor has been implicated in a number of processes related to neuronal development and plasticity^{16,17}, including neuronal survival¹⁸. Complete loss of NMDAR-mediated synaptic current is achieved by the disruption of the *NR1* (also known as *Grin1*) gene¹⁵, which encodes an essential subunit for functional NMDAR.

In most experiments described below, we injected a mixture of CAG–GFP/Cre and CAG–monomeric red fluorescent protein 1 (mRFP1) into the dentate gyrus of floxed *NR1* (fNR1) mice to analyse both *NR1* knockout (NR1KO: GFP/Cre, mRFP1 double-positive or GFP/Cre-positive only) and wild-type (mRFP1-positive only) new neurons in the same mice (Fig. 2a, b). We did not find any systematic difference in morphology between NR1KO and wild-type new neurons at any time point examined (Fig. 2b). A number of dendritic spines, major sites of glutamatergic synapses, were formed in NR1KO new neurons by 4 weeks after virus injection (Fig. 2c). Using electron microscopy, we found that these dendritic spines made contact with axon terminals and formed mature synapses (Fig. 2d), indicating that these new neurons are integrated into existing circuits. The temporal pattern of neuronal marker expression¹⁰—double-positive for doublecortin (DCX) and neuronal nuclei (NeuN, also known as NEUN60) at 14 days (Fig. 2e);

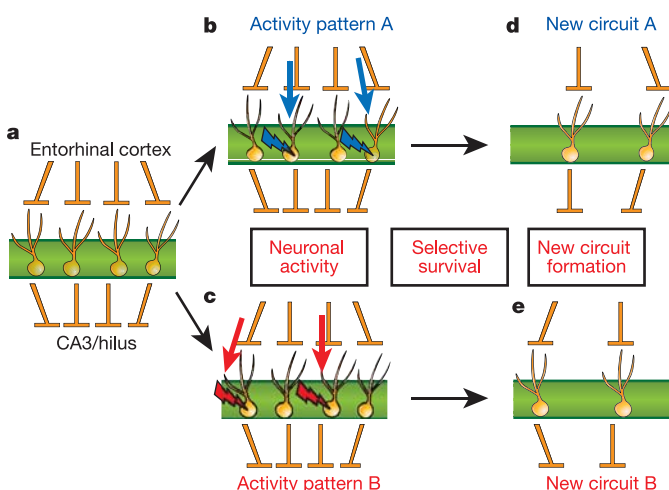


Figure 1 | Information-specific formation of new circuits by selective survival/death of new neurons: a hypothesis. After new neurons are generated (a), the different patterns of input activity (red and blue arrows) to the dentate gyrus, which are associated with different information related to the animal's experience, activate different subsets of new neurons (b, c). Then, the activated new neurons are selected for survival (d, e). Thus, the resulting new circuits are selectively determined by input activity and represent the information associated with the input activity.

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thereafter NeuN stays but DCX is downregulated (data not shown)—in GFP/Cre-positive neurons in wild-type and fNR1 mice was intact, indicating that the expression of GFP/Cre itself does not affect the maturation of new neurons and that NMDAR is not essential in the general mechanism of neuronal maturation.

To assess the efficiency of the Cre-mediated recombination with the retrovirus, CAG-GFP/Cre was injected into the dentate gyrus of adult, ROSA26-GFP reporter mice (also known as Gt(ROSA)26Sor^{tm2Sho}) (Fig. 2f). Because GFP/Cre is localized in the nucleus, GFP signal in the dendrites of new neurons in these ROSA26-GFP reporter mice indicates the occurrence of recombination. The reporter GFP gene was expressed in almost all Cre-positive neurons (7 days after injection 97.5% have GFP in the dendrites for $n = 40$ GFP/Cre, DCX double-positive cells; 14 days after injection >99% have GFP for $n = 136$ cells; 21 days after injection 100% have GFP for $n = 8$ cells). Thus, Cre-mediated recombination using CAG-GFP/Cre is highly efficient.

To confirm that functional NMDAR is not expressed in GFP/Cre-positive neurons in fNR1 mice, we took advantage of the destructive effects of high concentrations of NMDA, an agonist of NMDAR,

which has been known to lead to the formation of dendritic beading¹⁹. We consistently observed dendritic beading (2 out of 5 neurons at 7 days after injection, all neurons at 14 days ($n = 5$) and 21 days ($n = 6$)) in mRFP1-positive wild-type new neurons within 30 min of bath application of 500 μ M NMDA in the presence of 1 μ M tetrodotoxin (TTX), 20 μ M 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 5 μ M glycine (Fig. 2g). In contrast, none of the GFP/Cre-positive neurons (7 days, $n = 15$ neurons; 14 days, $n = 7$; 21 days, $n = 5$) showed dendritic beading 30 min after NMDA application (Fig. 2g), demonstrating that Cre-mediated gene knock-out is completed in most, if not all, GFP/Cre-positive neurons by 1 week after virus injection. Indirect excitation was blocked by antagonists and surrounding neurons are essentially all normal (Cre-negative), so it is unlikely that this difference between GFP/Cre-positive and -negative new neurons is caused by an indirect mechanism. Additionally, we performed whole-cell recordings from GFP-positive and GFP/Cre-positive new neurons 13–15 days after virus injection ($n = 4$ each; Fig. 2h, i). GFP-positive, but not GFP/Cre-positive, new neurons generated large inward currents in response to bath application of 100 μ M NMDA (Fig. 2j).

Next, we measured the densities of wild-type and NR1KO new neurons in fNR1 mice 1–6 weeks after virus injection in fNR1 mice. Consistent with previous studies using 5-bromo-2-deoxyuridine (BrdU)^{10,20,21}, we found a significant decrease in the density of wild-type new neurons within 3 weeks of virus injection (Fig. 3a; $n = 8$ mice for all groups; $P < 4 \times 10^{-7}$ for analysis of variance, ANOVA; $P < 0.02$ for Fisher's LSD test between the 7-days group and all other groups). The decrease in the density of NR1KO new neurons was significantly higher than that of wild-type new neurons (Fig. 3a). The relative decrease in the normalized density of NR1KO new neurons compared with wild-type new neurons in fNR1 mice occurred exclusively between 2 and 3 weeks after virus injection. The NMDAR-independent decrease in the density of new neurons less than 2 weeks after virus injection indicates the existence of an additional survival mechanism during this period. To exclude the

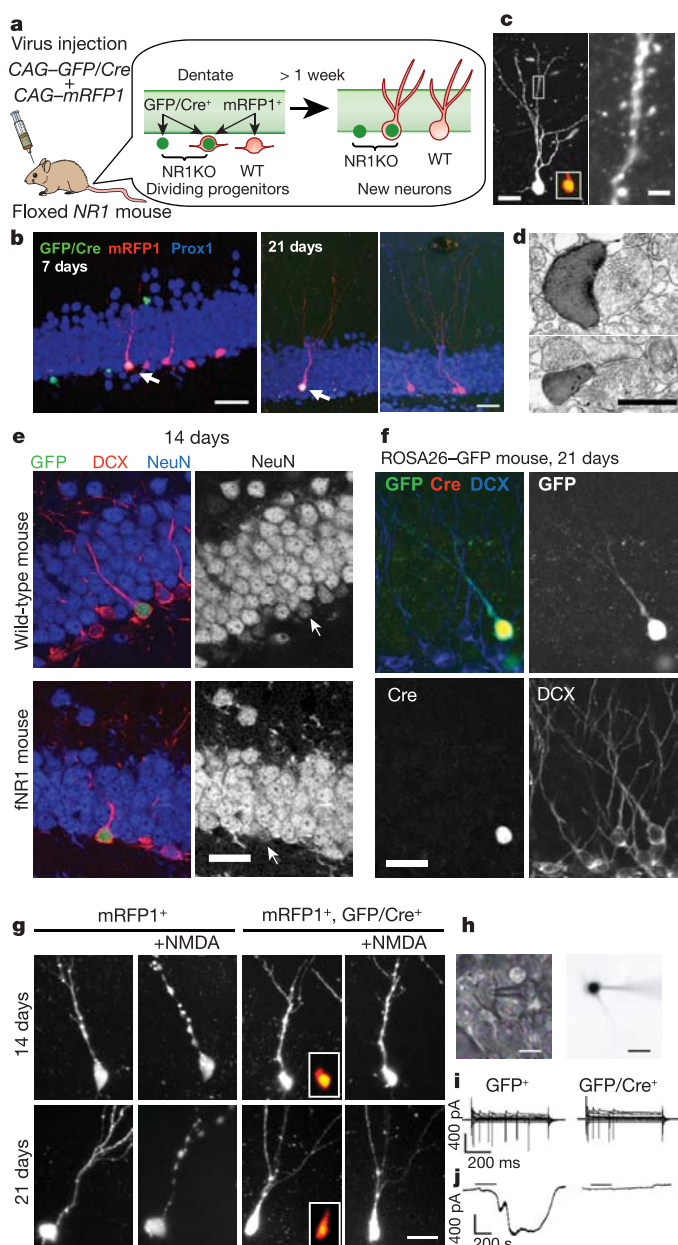


Figure 2 | Retrovirus-mediated gene knockout technique. **a**, A schematic of the experimental principle. The viral DNA is integrated into dividing neuronal progenitor cells, which become either GFP/Cre-positive (GFP/Cre⁺; green) only, mRFP1-positive (mRFP1⁺; red) only or double-positive. In GFP/Cre-positive cells, Cre-mediated recombination occurs, and they become NR1KO cells. These cells eventually become neurons. **b**, GFP/Cre-positive (green), mRFP1-positive (red) and double-positive (arrows) new neurons at 7 and 21 days after virus injection. Blue, Prox1 immunostaining. **c**, Dendritic spines formed on GFP/Cre-positive new neurons 4 weeks after virus injection in fNR1 mice. Grey rectangle in the low-magnification image (left) corresponds to the high-magnification image (right). The inset shows colocalization (yellow) of GFP/Cre and mRFP1 (red). **d**, Electron micrographs showing that dendritic spines on a GFP/Cre-positive new neuron (dark profiles) receive synapses. **e**, GFP/Cre-positive new neurons in fNR1 and wild-type mice. Red, DCX; Blue, NeuN (arrows in right panels), at 14 days. **f**, Cre-mediated recombination using retrovirus. Because GFP/Cre is localized in the nucleus, GFP signal in the dendrites (top right panel) of new neurons in ROSA26-GFP reporter mice indicates the occurrence of recombination. **g**, The absence of functional NMDAR in GFP/Cre-positive new neurons in fNR1 mice. NMDA application caused dendritic beading in wild-type (mRFP1⁺) new neurons but not in GFP/Cre-positive (mRFP1⁺, GFP/Cre⁺) new neurons in fNR1 mice. The insets show colocalization (yellow) of GFP/Cre and mRFP1 (red). **h**, Differential interference contrast (left) and fluorescent (right) images of a recorded GFP/Cre-positive new neuron. Strong (in nucleus) and weak (in cytoplasm) fluorescence signals correspond to GFP/Cre and Alexa 594, respectively. **i**, Action potentials generated by GFP-positive (left) and GFP/Cre-positive (right) new neurons in response to depolarization. **j**, Large inward current was evoked by bath application of NMDA in GFP-positive (left) but not in GFP/Cre-positive new neurons (right). Horizontal bars above the traces indicate the timing of bath application. Scale bars: 30 μ m in **b** and **e**; 20 μ m in **c** (left), **f** and **g**; 10 μ m in **h**; 2 μ m in **c** (right); 0.5 μ m in **d**.

possibility that this observed effect was due to a detrimental effect of GFP/Cre expression, we performed the same analysis at up to 4 weeks in wild-type mice. We found no difference in the normalized densities of mRFP1-positive only and GFP/Cre-positive new neurons at any time point (Fig. 3b; $P > 0.39$, $n = 6$ –7 mice for all groups, two-sided t -test).

To confirm that the decrease in the density of new neurons observed in fNR1 mice was due to neuronal death, we examined the activation of caspase 3 (refs 22, 23). In this experiment, we injected CAG–GFP or CAG–GFP/Cre virus into fNR1 mice. We found immunoreactivity for activated caspase 3 in a fraction of both GFP-positive and GFP/Cre-positive new neurons 18 days after virus injection ($n = 154$, NR1KO; $n = 390$, wild-type) but observed no immunoreactivity either at 14 or 21 days after injection ($n = 90$ –301 neurons; Fig. 3c, d). Occasionally, we found activated caspase 3-positive, DCX-positive neurons without GFP or GFP/Cre (Fig. 3e), indicating that the death of young new neurons was a physiological event that occurred without viral transduction.

Activity-dependent processes refine the formation of functional neural circuits in the developing brain²⁴. Some of these have been characterized as competitive processes^{24,25}. We hypothesized that new

neurons compete for survival through the activation of their own NMDAR. To test this possibility, we combined the retrovirus-mediated single-cell gene knockout technique with the injection of an NMDAR antagonist, 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP). CPP blocks, or reduces, NMDAR activation in wild-type neurons but not in NR1KO neurons because of the complete absence of functional NMDAR in NR1KO neurons. Therefore, the injection of CPP reduces the difference between the levels of NMDAR activation in wild-type and NR1KO neurons (Fig. 4a). If there were a competition for survival dependent on the relative levels of NMDAR activation, CPP injection should increase the survival of NR1KO new neurons, which was originally low in normal conditions because of the large difference in NMDAR activation from wild-type neurons (Fig. 4a). We injected a mixture of CAG–GFP/Cre and CAG–mRFP1 into fNR1 mice and then injected CPP (10 mg per kg body weight intraperitoneally) daily for 7 days during the

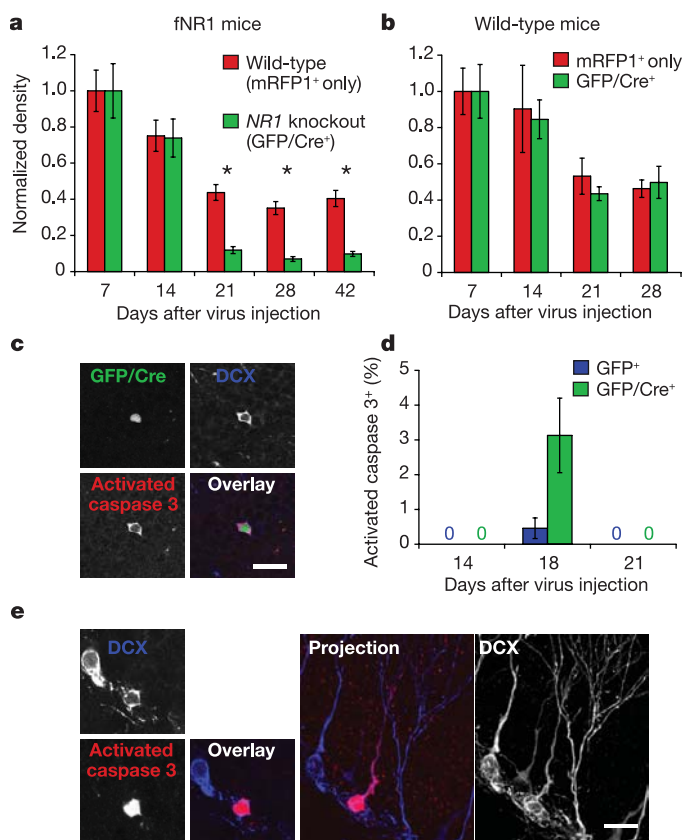


Figure 3 | The absence of functional NMDAR reduces the survival rate of new neurons. **a**, The survival rate of wild-type (mRFP1-positive only) and NR1-knockout (GFP/Cre-positive only and double-positive) new neurons in fNR1 mice ($*P < 0.0002$, two-sided t -test, $n = 8$ mice for each time point). **b**, The survival rate of mRFP1-positive only and GFP/Cre-positive (including GFP/Cre-positive only and double-positive) new neurons in wild-type (C57BL/6) mice. **c**, The presence of activated caspase 3 in a small population of NR1KO new neurons at 18 days after virus injection. The images are from a single focal plane. Red, activated caspase 3; blue, DCX; green, GFP/Cre in an overlay image. **d**, The percentage of activated caspase 3-positive new neurons. **e**, Endogenous activation of activated caspase 3 in a small population of new neurons without virus transduction. Left panels are from a single focal plane. Right panels are projected images from Z-series. Scale bars, 30 μ m. The data are shown as mean $\pm$ s.e.m. in **a**, **b** and **d**.

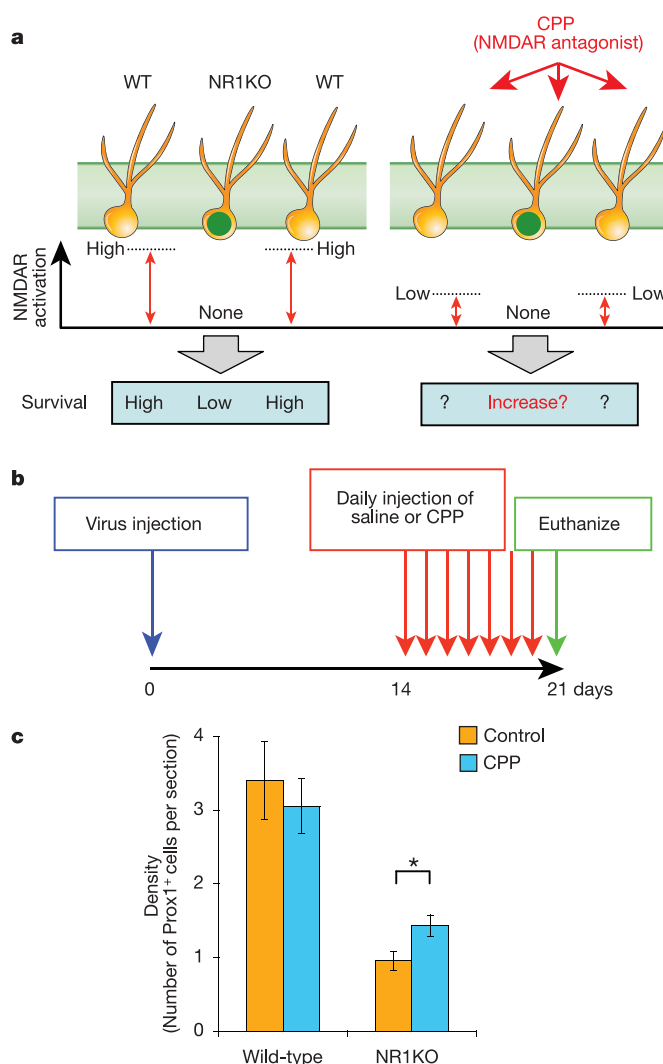


Figure 4 | Blockade of NMDAR in surrounding neurons increases the survival of NR1KO new neurons. **a**, A schematic showing the concept of the experiment. The injection of an NMDAR antagonist, CPP, reduces the difference in the level of NMDAR activation between NR1KO new and surrounding wild-type (WT; new or old) neurons. If the survival regulation were competitive or determined by the relative level of NMDAR activation, the survival of NR1KO new neurons should be increased by the injection of CPP. **b**, The experimental timeline. **c**, The density of wild-type and NR1KO new neurons in saline- or CPP-injected fNR1 mice. The data are shown as mean $\pm$ s.e.m. ($*P < 0.03$, $n = 9$ control mice; $n = 8$ CPP mice; two-sided t -test).

third week after virus injection (Fig. 4b)—the period in which NMDAR-dependent survival/death of new neurons occurs. Whereas the survival of wild-type new neurons was unchanged ($P > 0.6$, two-sided t -test), CPP injection increased the survival of NR1KO neurons by $\sim 50\%$ (Fig. 4c), indicating that the survival of new neurons is competitively regulated depending on the relative levels of NMDAR activation (Supplementary Fig. 2), although it is not clear from this analysis whether new neurons compete with surrounding new neurons or mature neurons.

Although the death of new neurons and the expression of functional NMDAR begin earlier, the NMDAR-dependent survival/death is restricted to the third week after neuronal birth. Thus, neuronal activity in the dentate gyrus within a short, critical time window seems to determine the survival of new neurons and the resulting formation of new circuits. This critical period is associated with a high degree of morphological change in new neurons, including synapse formation^{13,26,27}. This temporal association suggests that the NMDAR-dependent regulation of survival is closely related to synapse formation, although synapse formation itself does not require functional NMDAR. In many parts of the developing brain, the death of immature neurons has been found to occur around the peak period of synaptogenesis^{12,28}, which parallels the maturational stage of new neurons during the critical period for the NMDAR-dependent survival/death that we observed in the adult brain. Therefore, NMDAR-dependent, cell-specific regulation of neuronal survival might be a general mechanism involved in the formation of functional circuits in the developing and adult brain.

Our data do not exclude the possible involvement of extra-synaptic NMDAR. However, this is unlikely because previous studies in other neuronal populations have shown that the activation of extra-synaptic NMDAR induces neuronal death in pathological conditions, whereas synaptic NMDAR promotes neuronal survival in physiological conditions^{29,30}. Thus, the construction of new circuits through selective survival/death of new neurons is probably regulated by synaptic activity, and in an information-specific manner. This information-specific construction of new circuits could be a unique mechanism by which newborn neurons in adult dentate gyrus contribute to information storage related to learning and memory.

METHODS

Retroviral vectors. Retroviral vectors were modified from CAG–GFP¹³ by replacing the sequence encoding GFP with other cDNAs. Preparation of concentrated retrovirus was previously described¹³.

Treatments. Retroviral vectors ($\sim 1 \times 10^7$ particles per ml; 1.5 μ l) were stereotactically injected into the dentate gyrus of 6–8-week-old mice under anaesthesia with ketamine/xylazine solution. To inject a mixture of CAG–GFP/Cre and CAG–mRFP1, two viral preparations were mixed (1:1 in volume) and 1.5 μ l was injected. CPP (Tocris; 10 mg per kg body weight, intraperitoneally) was injected into fNR1 mice daily 14–20 days after virus injection.

Fluorescence microscopy. To examine the colocalization of fluorescence signals in fixed samples, we used a Radiance 2100 confocal microscope with a $\times 40$ oil-immersion objective lens (numerical aperture 1.30). For live imaging, we used the same confocal microscope with a $\times 60$ water-immersion objective lens (numerical aperture 0.90). Live slices were placed on an imaging chamber that was perfused at room temperature ($\sim 20^\circ\text{C}$) with magnesium-free artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 3 mM KCl, 2 mM CaCl_2 , 1.1 mM NaH_2PO_4 , 26 mM NaHCO_3 , and 10 mM dextrose and saturated with 95% O_2 and 5% CO_2 . On the basis of their position and morphology, we found mRFP1-positive or mRFP1; GFP/Cre-double-positive new neurons in the dentate gyrus. Then, to prevent indirect stimulation of the new neurons through synaptic activity from other neurons, 1 μ M TTX and 20 μ M DNQX were applied with bath application. After acquiring a Z-series of these new neurons, 500 μ M NMDA was applied with bath application. After 30 min, the second Z-series was acquired. In addition to NMDA, a coagonist of NMDAR, glycine (5 μ M), was applied to maximize NMDAR activation.

Density analysis of new neurons. To measure the density of fluorescently labelled new neurons, sections were selected evenly from anterior to posterior regions of the dentate gyrus. The numbers of mRFP1-positive only, GFP/Cre-positive only and double-positive new neurons—identified by prospero-related homeobox 1

(Prox1) immunoreactivity—were counted for each section. To calculate the density of new neurons, the number of new neurons was divided by the number of examined sections containing any fluorescently labelled cells. The examined sections without any fluorescent cells were not included in this calculation to avoid variability caused by the difference in diffusion of injected virus in the dentate gyrus. The densities of new neurons (mRFP1-positive only, GFP/Cre-positive only, and double-positive) were affected by the precise titre of viruses. Therefore, the absolute numbers of fluorescently labelled neurons were highly variable when different viral preparations were injected, even though we tried to measure and match virus titre precisely. However, with the same viral preparations, the densities of fluorescently labelled new neurons were reliable between mice. Therefore, we always used the same viral preparations for each experiment. For the same reason, for developmental analysis (Fig. 2a,b), the density at each time point was normalized by the density at 7 days to compare directly the decreasing rate of the density of new neurons transduced by different viruses (CAG–GFP/Cre and mRFP1).

Electrophysiology. Brain slices were prepared from 8–10-week-old fNR1 mice injected with CAG–GFP or –GFP/Cre 13–15 days before. GFP or GFP/Cre-positive cells were identified by their fluorescence. Recorded cells were filled with 5 μ M of Alexa Fluor 594 (Molecular Probes) through the recording pipette to verify that the recorded cell was indeed GFP-positive. To check the neuronal identity and viability of recorded cells, we examined whether recorded cells generated action potentials in response to membrane depolarization. NMDA (100 μ M) was applied in the presence of TTX (1 μ M), DNQX (20 μ M) and glycine (5 μ M) and in the absence of Mg^{2+} . We recorded from four GFP-positive and four GFP/Cre-positive neurons. Fluorescent images of recorded cells were taken after recordings were completed.

Electron microscopy. Four weeks after injection with CAG–GFP/Cre, 2–3-month-old mice were perfused with saline followed by a solution of phosphate buffer saline (PBS) containing 4% paraformaldehyde and 0.2% glutaraldehyde, and sections were cut at a thickness of 100 μ m. Cells were injected under a fluorescence microscope with 5% aqueous Lucifer yellow (Sigma). Slices were then incubated with 2.8 mM 3,3'-diaminobenzidine (DAB) tetrahydrochloride and 6 mM potassium cyanide and then irradiated under conventional epifluorescence using a 75-W Hg lamp and a fluorescein filter set to induce photoconversion of DAB tetrahydrochloride into an electron-dense residue. Slices were then post-fixed overnight in a solution of 3% glutaraldehyde and processed conventionally for electron microscopy. Serial sections were cut at a thickness of 35 nm and analysed with a JEOL 100CXII electron microscope at a $\times 48,000$ magnification.

A more detailed description of these methods is presented in the Supplementary Information.

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Author contributions A.T. conceived and performed the experiment, analysed the data and wrote the manuscript. V.M.S. performed the electrophysiological experiment. N.T. performed the electron microscopy experiment. C.Z. provided unpublished viral vectors and helped in the construction of a new viral vector. F.H.G. revised the manuscript and obtained the financial support.

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LETTERS

The cells and logic for mammalian sour taste detection

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Mammals taste many compounds yet use a sensory palette consisting of only five basic taste modalities: sweet, bitter, sour, salty and umami (the taste of monosodium glutamate)^{1,2}. Although this repertoire may seem modest, it provides animals with critical information about the nature and quality of food. Sour taste detection functions as an important sensory input to warn against the ingestion of acidic (for example, spoiled or unripe) food sources^{1–3}. We have used a combination of bioinformatics, genetic and functional studies to identify PKD2L1, a polycystic-kidney-disease-like ion channel⁴, as a candidate mammalian sour taste sensor. In the tongue, PKD2L1 is expressed in a subset of taste receptor cells distinct from those responsible for sweet, bitter and umami taste. To examine the role of PKD2L1-expressing taste cells *in vivo*, we engineered mice with targeted genetic ablations of selected populations of taste receptor cells. Animals lacking PKD2L1-expressing cells are completely devoid of taste responses to sour stimuli. Notably, responses to all other tastants remained unaffected, proving that the segregation of taste qualities even extends to ionic stimuli. Our results now establish independent cellular substrates for four of the five basic taste modalities, and support a comprehensive labelled-line mode of taste coding at

the periphery^{5–10}. Notably, PKD2L1 is also expressed in specific neurons surrounding the central canal of the spinal cord. Here we demonstrate that these PKD2L1-expressing neurons send projections to the central canal, and selectively trigger action potentials in response to decreases in extracellular pH. We propose that these cells correspond to the long-sought components of the cerebrospinal fluid chemosensory system¹¹. Taken together, our results suggest a common basis for acid sensing in disparate physiological settings.

A broad range of cell types, receptors and mechanisms have been proposed to mediate salt and acid sensing in taste receptor cells (TRCs)^{1–3}. These include the activation of epithelial sodium channels (ENaCs), acid-sensing ion channels (ASICs), potassium (K²P) channels, H⁺-gated calcium channels, as well as the involvement of Na⁺-H⁺ exchangers, TRPV pain receptors, and even acid inactivation of K⁺ channels^{1–3,12,13}. Notably, most of these proteins are broadly expressed in TRCs and other tissues. In contrast, we previously isolated and characterized the receptors for sweet, umami and bitter taste^{5–7,14–16}, and showed that each of these three taste modalities is mediated by highly selective receptor proteins expressed in distinct and independent populations of TRCs^{5–10}.

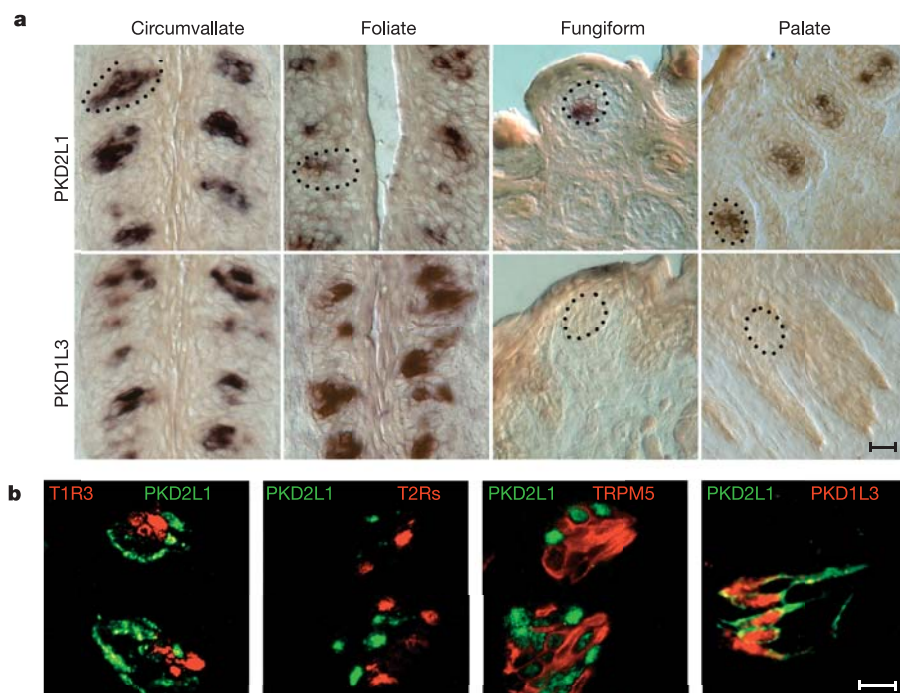


Figure 1 | PKD2L1 is expressed in a novel population of TRCs. *In situ* hybridization (PKD2L1, PKD1L3, T1Rs, T2Rs and TRPM5) and double-label fluorescent immunohistochemistry (PKD2L1) were used to examine the overlap in cellular expression of taste receptors, TRPM5, PKD2L1 and PKD1L3. **a**, *In situ* hybridization of PKD2L1 and PKD1L3 against circumvallate, foliate, fungiform and palate taste buds illustrating expression of PKD2L1 in subsets of TRCs of all taste buds, but lack of PKD1L3 in fungiform and palate TRCs. Dotted lines show the outline of sample taste buds. Scale bar, 25 μm. **b**, The first three panels show co-labelling with a PKD2L1 antisense RNA probe (green) and T1R3 (T1R, sweet and umami cells), a mixture of 20 T2Rs (bitter cells) and TRPM5 (sweet, umami and bitter cells), respectively. The last panel shows co-labelling with anti-PKD2L1 antibodies and an antisense PKD1L3 RNA probe. Note the absence of overlap between PKD2L1-expressing cells and those expressing sweet, umami or bitter receptors. However, PKD1L3 is always co-expressed with PKD2L1 in circumvallate and foliate papillae. Scale bar, 10 μm.

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Therefore, we reasoned that salt and sour taste should also be mediated by highly selective dedicated cells, and consequently expected the receptor proteins to be very exclusive in their expression pattern.

To identify novel taste receptors, we developed a multi-step bioinformatics and expression screening strategy. First, because sensory receptors are expected to be membrane proteins, approximately 30,000 mouse open reading frames (ORFs) were scanned for the presence of at least one putative transmembrane segment. Second, because taste receptors are predicted to be very restricted in their expression pattern, ORFs encoding candidate transmembrane proteins were cross-searched against mouse expressed sequence tag (EST) databases to eliminate those broadly expressed. Next, to identify the subset specifically enriched in taste tissue, ORFs selected as encoding transcripts infrequently represented in EST databases (~880 candidates) were used in polymerase chain reaction with reverse transcription (RT-PCR) reactions templated with messenger RNA from TRCs versus control tongue epithelium. Finally, given that our goal was to discover membrane proteins selectively expressed in subsets of TRCs (and ideally not in sweet-, bitter- or umami-sensing cells), we carried out detailed *in situ* hybridizations against taste papillae. Of 26 complementary DNAs used for *in situ* studies, five were found to robustly and selectively label subsets of TRCs. Figure 1 shows that one of these candidates,

PKD2L1, is expressed in TRCs of all taste papillae, including fungiform, circumvallate, foliate and palate taste buds.

PKD2L1 encodes a polypeptide displaying significant amino acid sequence similarity to PKD2 (ref. 4), a gene mutated in many cases of autosomal-dominant polycystic kidney disease^{17,18}. PKD2 proteins are members of the transient receptor potential (TRP) superfamily of ion channels¹⁹, and have been recently shown to function as non-selective cation channels when expressed in heterologous cells^{17,18,20}. Although the exact roles of PKD proteins remain unknown, they are believed to function as receptor-ion-channel complexes, often localized to ciliated compartments, and implicated in sensing extracellular signals (for example, in renal epithelial cells^{17,18}). We reasoned that if PKD2L1 has a specific role in taste it should be expressed in subpopulations of TRCs with unique functional characteristics. To determine which type of TRCs expresses PKD2L1, we performed double labelling experiments with sweet, umami and bitter taste receptors (T1Rs and T2Rs), as well as TRPM5, the transduction channel of sweet-, bitter- and umami-sensing cells⁸. Our results (Fig. 1) established that PKD2L1 is expressed in cells distinct from those mediating sweet, umami and bitter taste (see also ref. 21).

Mammalian TRCs project specialized apical microvilli to the taste pore, the site of interaction between tastants and taste receptor proteins. All known taste receptor proteins localize to and function in this TRC compartment^{1,5-7,14,16,22}. Therefore, we would expect

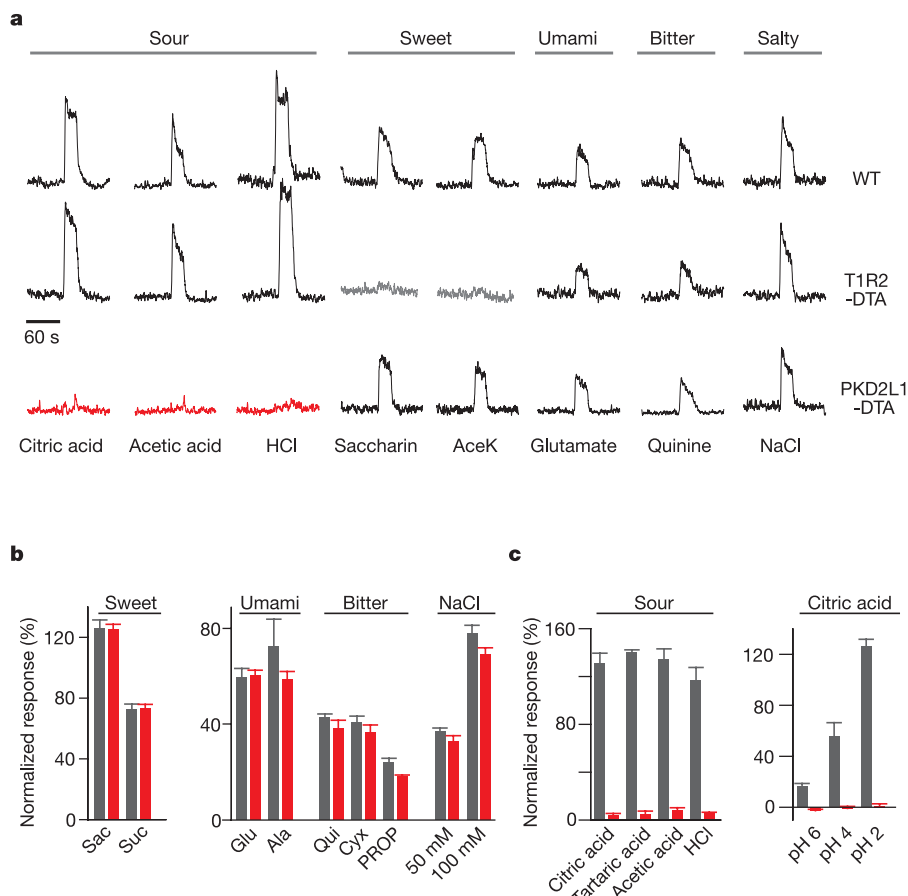


Figure 2 | PKD2L1-expressing TRCs are the mediators of sour taste.

a, Targeted expression of DTA to selective populations of TRCs produces animals with selective deficits in taste responses. Wild-type mice (WT) show robust neural responses to sour, sweet, umami (amino acid), bitter and salty tastants. However, ablation of sweet cells (T1R2-DTA) generates animals with a marked loss of sweet taste (middle panel). In contrast, ablation of PKD2L1-expressing cells eliminates responses to all acid stimuli (bottom panel). Notably, responses to all other taste qualities remain unimpaired in the DTA-expressing animals. Integrated chorda tympani responses are

shown (see Methods). **b**, Average neural responses of animals lacking PKD2L1-expressing cells to an expanded panel of tastants; note normal responses to sweet, umami, bitter and salt stimuli. Wild type, black bars; PKD2L1-DTA, red bars; values are means $\pm$ s.e.m. ($n = 5$).

c, Quantification of acid responses of wild-type and PKD2L1-DTA animals. The values are means $\pm$ s.e.m. ($n = 6$). Only the differences in acid responses are significant between wild-type and PKD2L1-DTA mice ($P < 0.00001$).

bona fide candidate receptors to also be enriched in the taste pore. We generated antibodies to PKD2L1 and used them in immunofluorescence staining of tongue tissue sections. Examination of circumvallate, foliate and fungiform papillae demonstrated that PKD2L1 protein is indeed enriched in the apical surface of TRCs, with the antibodies robustly labelling the taste pore region (Supplementary Fig. 1). These results implicate PKD2L1 as part of the taste sensing machinery.

PKD2 isoforms often require PKD1 proteins for functional expression at the cell surface^{17,18,20}. The mammalian genome contains four members of the PKD1 family: PKD1, PKD1L1, PKD1L2 and PKD1L3 (refs 17, 18). We performed *in situ* hybridization studies with gene-specific probes representing each family member, and determined that PKD1L3 is specifically co-expressed with PKD2L1 in circumvallate and foliate TRCs (Fig. 1, see also ref. 21). We also generated antibodies to PKD1L3 and demonstrated selective co-expression with PKD2L1 in non-TRPM5-expressing cells of the circumvallate and foliate taste buds (Fig. 1 and Supplementary Fig. 1). Surprisingly, PKD1L3 transcript or protein is not detectable in fungiform or palate taste buds, suggesting that a different partner may be expressed in those TRCs.

To dissect the function of PKD2L1-expressing cells in the tongue, we engineered mice where these cells were genetically ablated by targeted expression of attenuated diphtheria toxin²³ (DTA). To validate this approach as a means of uncovering TRC function, we first generated mice in which T1R2-regulatory sequences were used to target DTA expression²⁴ (see Supplementary Fig. 2). T1R2 is an essential subunit of the sweet receptor heterodimer (composed of T1R2 plus T1R3), and the selective ablation of these cells should generate animals with a specific loss of sweet taste^{6,9,10,16}. To investigate the taste responses of the genetically modified mice, we recorded tastant-induced action potentials from nerves innervating taste cells of the tongue; this physiological assay monitors the activity of the taste system at the periphery, and provides an accurate and reliable measure of TRC function. Indeed, animals expressing DTA in T1R2 cells have an extraordinary loss of sweet, but importantly retain umami, bitter, sour and salty tastes (Fig. 2). These results further substantiate the exquisite segregation of taste modalities at the

periphery, and demonstrate the utility of using DTA-mediated ablation of TRCs as a strategy for dissecting taste system function. Next, we engineered animals in which the *Pkd2l1* gene was used to target Cre recombinase into PKD2L1-expressing cells (see Supplementary Figs 2 and 3). These mice were crossed to the conditional DTA lines²⁴, and double-positive progeny were scrutinized both for the specificity and efficiency of killing, as well as the integrity of taste buds. We checked for the expression of T1Rs, T2Rs and TRPM5 in control and DTA-expressing animals, and found no significant differences in the number or distribution of T1R- or T2R-positive cells between wild-type and ablated taste tissue^{5–8,14,25} (Supplementary Fig. 2). In contrast, the DTA-targeted mice had a profound and practically complete loss of PKD2L1-expressing TRCs in the tongue (Supplementary Fig. 2). Notably, genetic ablation of the PKD2L1-expressing cells produces animals with a devastating loss of sour taste (Fig. 2). Responses to all acid tastants, including citric acid, HCl, tartaric acid and acetic acid are completely abolished, with no significant activity over a range of five orders magnitude of proton concentrations. However, responses to sweet, umami, bitter or salty tastants remain indistinguishable from wild-type control animals. These results firmly establish PKD2L1-expressing cells as the sour taste sensors, and further substantiate a model of coding at the periphery in which individual taste modalities operate independently of each other.

Acid sensing is important not only in the taste system, but also for monitoring the functional state of body fluids, including the internal milieu of the brain. This is particularly well studied in the central and peripheral control of respiration, where pH sensing is the principal mechanism for monitoring CO₂ levels in the blood and cerebrospinal fluid (CSF)^{11,26,27}. Thus, we wondered whether PKD2L1 might be expressed in additional cell types, and if so, whether such cells may also be involved in pH sensing in other physiological systems.

We carried out *in situ* hybridization and antibody staining experiments with PKD2L1 on a wide range of other tissues and identified a singular additional domain of expression: a discrete population of neurons surrounding the central canal of the spinal cord, through its entire length, from its origin in the brain stem to its end around the cauda equina (Fig. 3). Notably, these neurons send processes into the

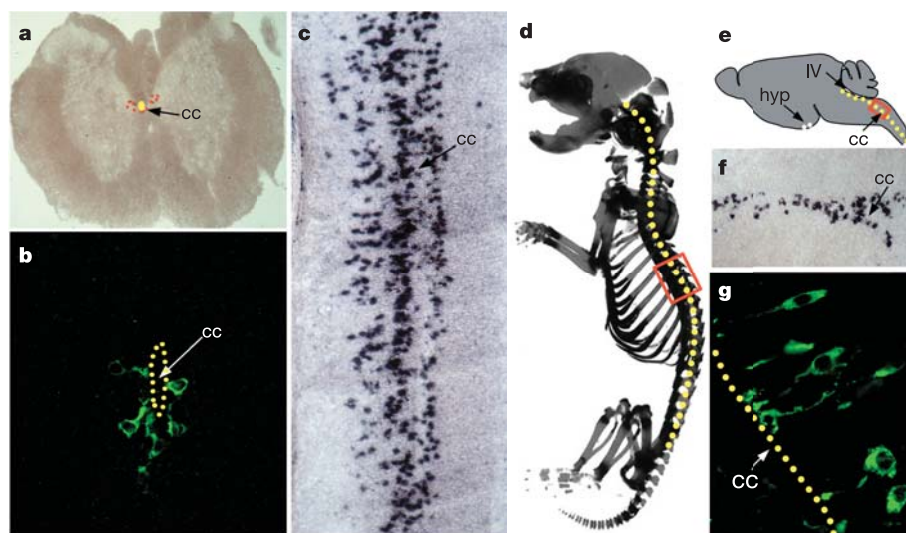


Figure 3 | PKD2L1 is expressed in neurons contacting the central canal of the spinal cord. **a**, *In situ* hybridization with PKD2L1-specific probes to a coronal section of the spinal cord; signals are pseudo-coloured in red. **b**, High-magnification staining with anti-PKD2L1 antibodies reveals a population of PKD2L1-expressing neurons surrounding the central canal of the spinal cord (cc; highlighted by yellow dots in all panels). **c**, *In situ* hybridization with PKD2L1-specific probes on a sagittal section of a P1 mouse. The PKD2L1-expressing cells are found throughout the entire length

of the spinal cord. **d**, Model mouse skeleton illustrating the approximate area (red box) of the *in situ* hybridization performed in **c**. **e**, **f**, PKD2L1 expression extends through the brain stem and into the IV ventricle (IV). There is also a very small group of positive cells in the hypothalamus (hyp; data not shown). **g**, Immunofluorescent staining with anti-PKD2L1 antibodies. PKD2L1-expressing neurons project into the central canal; note robust expression of PKD2L1 receptors at the terminals.

central canal, indicating that they may function as chemoreceptors sensing the internal state of the CSF (Fig. 3b, g; see also ref. 11). Given their anatomical distribution and cellular morphology, we reasoned that these cells might be part of the homeostatic circuitry responsible for monitoring and reporting the pH of the CSF. This hypothesis predicts that these neurons should trigger action potentials in response to acid stimulation. Therefore, we engineered mice where a green fluorescent protein (GFP) reporter was targeted to PKD2L1-expressing cells, and performed patch-clamp recordings from GFP-labelled cells in a spinal cord slice preparation²⁸. We anticipated some notable differences in the behaviour of these cells compared to TRCs—whereas the taste system is tuned to respond to acid stimulation in the range of multiple pH units (that is, pH 2–5), we expected the CSF monitor cells to respond to pH changes within a range of a few tenths of deviation from pH 7.4. Indeed, Fig. 4 shows that the PKD2L1-expressing neurons display exquisite sensitivity and selectivity to pH stimulation. Exposure to test solutions between pH 6.5 and 7.4 evoked a marked, dose-dependent and reversible increase in action potential frequency (Fig. 4 and data not shown). In contrast, the same acid stimuli have no significant impact on the response of control (unlabelled) cells, even after exposure to pH as

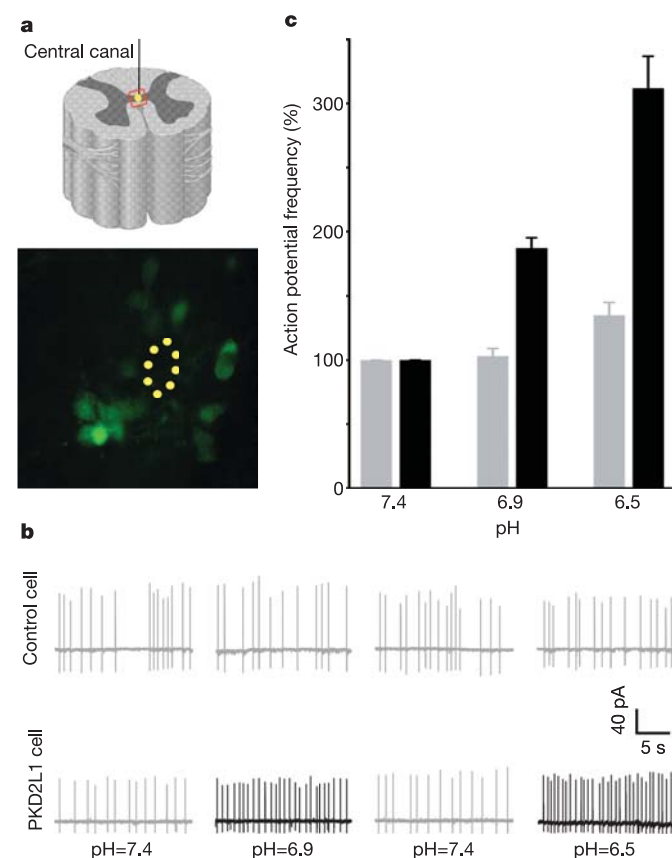


Figure 4 | PKD2L1-expressing neurons of the central canal fire action potentials in response to pH stimulation. Spinal cord neurons were patched using a loose patch configuration²⁸, tested for the presence of basal activity and recorded in the cell-attached configuration. **a**, GFP-expressing (PKD2L1-positive cells) or unlabelled (control) cells were examined for pH responses. **b**, Responses of a sample GFP-labelled or unlabelled neuron to test solutions under the indicated regime; shown are trains of spikes in a window of ~25 s. **c**, Data were analysed by examining records of ~4 min at each pH condition. Basal activity ranged between 1 to 5 Hz. Note the marked increases in pH-evoked firing frequency in GFP-labelled (black bars) neurons versus unlabelled (grey bars) cells ($P < 0.001$). A minimum of eight GFP-labelled and five unlabelled cells were characterized for each stimulus. The values are means $\pm$ s.e.m. normalized to basal activity at pH 7.4 (taken as 100%).

low as 6.5 (lower pH values triggered irreversible damage to the slice preparation).

Most of the known CSF-contacting neurons in mammals project ciliated dendrites into the CSF, where they are proposed to sense fluid flow, pressure, pH or the composition of the CSF¹¹. Our demonstration that PKD2L1-expressing cells of the spinal cord selectively fire in response to minor changes in proton concentration strongly suggests that they function as sentinels of cerebrospinal and ventricular pH. Collectively, these results uncover an entirely unexpected role for members of the PKD family of proteins, offer a new perspective into the potential importance of PKD2 proteins in health and disease, and bring forth a surprising unity in the cellular basis of pH sensing in very different physiological systems. In the future, it will be of interest to develop an activity assay for PKD2L1 to establish the molecular mechanism of acid activation, to study the phenotype of *Pkd2l1* knockout animals, and determine whether PKD2L1 functionally associates or interacts with different partners in different cells types. In this regard, it would be worth exploring whether the differences in pH sensitivity between the tongue and spinal cord might be due to differences in PKD2L1–receptor complex composition.

The nature of the mammalian sour taste receptor and sour-sensing TRCs has been fertile ground for speculation over the years. A wide range of cell types, receptors and even receptor-independent mechanisms have been proposed to mediate acid detection in the tongue^{1–3}. The results presented here establish that sour taste, much like our previous findings for sweet, umami and bitter taste, is mediated by a unique cell type, independent of all other taste qualities. Furthermore, our demonstration that sour-less mice have normal salt responses shows that salt taste is also mediated by independent TRCs. Together, these results impose a considerable revision of the current views of taste representation at the periphery, and make a compelling case for a labelled-line mode of coding across all five taste modalities and taste receptor cell types.

METHODS

Molecular cloning of PKD2L1. We used a strategy that combined bioinformatics and differential screening to isolate genes specifically expressed in TRCs. Mouse genomic sequence information was obtained from Ensembl Mm.30 (<http://www.ensembl.org>). Approximately 30,000 predicted protein sequences were screened for the presence of at least one putative transmembrane segment, using both TMHMM server version 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) and f₂TMHMM (San Diego Supercomputer centre, <http://www.sdsc.edu/pb/Group.html>). The cDNA sequence for each candidate membrane protein was then extracted from NCBI (http://www.ncbi.nlm.nih.gov/blast/blastcgihelp.shtml#nucleotide_databases) and used to screen EST databases (<http://www.ncbi.nlm.nih.gov/dbEST/index.html>). Only EST hits with e -values of less than or equal to e^{-100} were considered in our analysis. A total of 884 genes expressed in three tissues or less were chosen for PCR reactions with cDNA prepared from taste papillae mRNA (circumvallate and foliate) and from surrounding non-taste epithelial tissue (non-taste control). To maximise specificity of the PCR reactions, primer sets often included unique 3' untranslated region (UTR) sequences (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). A total of 98 genes showed selective enrichment in taste versus non-taste tissue, and of these five were robustly expressed in subsets of TRCs. Full-length clones were isolated from mouse taste cDNAs and/or cDNA libraries²².

In situ hybridization and immunostaining. *In situ* hybridization and immunostaining were as described previously^{8,22}. Anti-peptide antibodies to PKD2L1 (KLKMLERKGLSPGMGE) and PKD1L3 (DFQEADNYCHAQRGLAHT) were generated in rabbits and purified as described previously⁸.

Transgenic animals. Transgenic lines were produced by pronuclear injection of zygotes from FVB/N or CB6 (BALB/c $\times$ C57BL/6 hybrids) mice. The PKD2L1-IRES-Cre construct was generated in RP23-297K23 and the T1R2-IRES-Cre in RP23-348G10 (<http://bacpac.chori.org/>). Recombination was carried out exactly as described previously²⁹. Z/EG reporter lines³⁰ were obtained from Jackson Laboratories, and Rosa26-flox-lacZ-flox-DTA animals²⁴ were a gift of D. Riethmacher.

Nerve recordings. Lingual stimulation and recording procedures were performed as previously described^{7,9}. All data analyses used the integrated response over a 25-s period immediately after the application of the stimulus. Tastants

used for nerve recordings were: 10 mM, 60 mM acesulfame K (AceK); 10 mM, 60 mM sodium saccharin (saccharin); 300 mM sucrose; 30 mM monopotassium glutamate plus 1 mM inosine monophosphate (Glu); 30 mM L-alanine plus 1 mM inosine monophosphate (Ala); 10 mM quinine hydrochloride (Qui); 100 μ M cycloheximide (Cyx); 10 mM 6-*n*-propyl-2-thiouracil (PROP); 50 mM, 100 mM sodium chloride (NaCl); 10 mM, 50 mM citric acid; 10 mM, 50 mM tartaric acid; 50 mM, 500 mM acetic acid; pH 2 hydrochloric acid (HCl); 10 mM citric acid pH 2, 4 and 6. The mean response to 60 mM AceK was used to normalize responses to each experimental series in the wild-type and PKD2L1-DTA animals (Fig. 2b, c).

Spinal cord slice recordings. Electrophysiological experiments were performed on P1–P4 mice as previously described²⁸. All incubations included 10 μ M CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) and 50 μ M AP5 (D(-)-2-amino-5-phosphonopentanoic acid). Spinal cord slices 250–300- μ m thick were generated using a Vibratome 3000 Plus at 0–4 °C in a modified Ringers' solution (0.5 mM CaCl₂, 3.7 mM MgSO₄). After at least a 1-h recovery period, slices were transferred to a recording chamber and perfused with oxygenated Ringers' solution (pH 7.4) at room temperature. Cell-attached patch-clamp recordings from GFP-labelled and unlabelled cells were performed using an EPC-10/2 amplifier and Patchmaster software (HEKA Elektronik). Slices were stimulated with a solution containing 140 mM NaCl, 3 mM KCl, 1.3 mM MgSO₄, 2.5 mM CaCl₂, 10 mM glucose, 10 mM HEPES at various pH values (7.4, 6.9, 6.5). Eight out of eight GFP-labelled cells showed pH-dependent increases in action potential frequency.

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The nitrate/proton antiporter AtCLCa mediates nitrate accumulation in plant vacuoles

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Nitrate, the major nitrogen source for most plants, is widely used as a fertilizer and as a result has become a predominant freshwater pollutant. Plants need nitrate for growth and store most of it in the central vacuole¹. Some members of the chloride channel (CLC) protein family, such as the torpedo-fish CLC-0 and mammalian CLC-1, are anion channels^{2,3}, whereas the bacterial CLC-ec1 and mammalian CLC-4 and CLC-5 have recently been characterized as Cl^-/H^+ exchangers with unknown cellular functions^{4–6}. Plant members of the CLC family are proposed to be anion channels^{7,8} involved in nitrate homeostasis⁹; however, direct evidence for anion transport mediated by a plant CLC is still lacking. Here we show that *Arabidopsis thaliana* CLCa (AtCLCa) is localized to an intracellular membrane, the tonoplast of the plant vacuole, which is amenable to electrophysiological studies, and we provide direct evidence for its anion transport ability. We demonstrate that AtCLCa is able to accumulate specifically nitrate in the vacuole and behaves as a NO_3^-/H^+ exchanger. For the first time, to our knowledge, the transport activity of a plant CLC is revealed, the antiporter mechanism of a CLC protein is investigated in a native membrane system, and this property is directly connected with its physiological role.

Seven members of the CLC family have been identified in the *A. thaliana* genome⁸ (M. Vinauger, C. Lurin and G.E., unpublished data). Genetic studies have indicated that these proteins have a function in nitrate homeostasis^{9–11}. Disruption of the *AtCLCa* gene leads to nitrate under-accumulation in the two independent T-DNA insertion knockout alleles, *clca-1* (ref. 9) and *clca-2* (M. Allot, B. Bernasconi and G.E., unpublished data), indicating an involvement of AtCLCa in nitrate storage. The subcellular localization for plant CLC proteins is mitochondrial for tobacco CLCNt-1 (ref. 12) and chloroplastic for spinach CLC-f (ref. 13), whereas AtCLCa is proposed (albeit without experimental evidence) to reside in the vacuolar membrane⁹.

To determine the subcellular localization of the AtCLCa protein, green fluorescent protein (GFP) fusion proteins were transiently expressed in protoplasts from *Arabidopsis* cell suspensions. Confocal microscopy studies revealed a green fluorescent labelling that coincided with the tonoplast (Fig. 1a). Vacuoles were extracted from mesophyll cells of *A. thaliana* leaves. A western blot analysis of vacuolar membrane proteins confirmed the tonoplast localization of AtCLCa and provided evidence for the absence of AtCLCa protein in both knockout mutants (Fig. 1b).

After the vacuolar localization of AtCLCa was established, the patch-clamp technique was used to investigate anionic currents across the tonoplast of mesophyll cells. In the whole-vacuole configuration (voltage is with reference to the cytosolic side of the membrane¹⁴, therefore positive currents represent anions entering

the cytosol) under bi-ionic conditions (NO_3^- in the vacuolar lumen, Cl^- in the cytosol), we identified an anion current (Fig. 2a) that was present in 100% of patched vacuoles ($n > 60$). Under these conditions, the current was outward rectifying and showed two parts: a time-dependent part with a half-activation time of 603 ± 62 ms at 43 mV and an instantaneous part. The total current (sum of time-dependent plus instantaneous parts) corresponded to a current density of 8.7 ± 1.1 pA pF⁻¹ (Fig. 2b) at 43 mV. Measurements of the reversal potentials (E_{rev}) either using a tail protocol, addressing specifically the time-dependent current, or directly from the I - V curves yielded similar values of -45.6 ± 0.4 mV (data not shown) and -43.3 ± 1.4 mV (Fig. 2b), respectively. To characterize further this current, we substituted the vacuolar NO_3^- with an equal amount of Cl^- (Fig. 2a, b). The total current density decreased to 1.1 ± 0.1 pA pF⁻¹ at 36 mV, and the E_{rev} shifted to -4 ± 6 mV. Both time-dependent and instantaneous parts have the same E_{rev} and were similarly affected by this change in ionic conditions. The agreement between these measurements indicates that both parts are mediated by the same NO_3^- -selective transport system (Fig. 2b) and confirms that these parts are linked. Taken together, these results identify a novel voltage-dependent anion current with high selectivity for nitrate over chloride on the luminal side.

To test whether the nitrate-selective current is mediated by

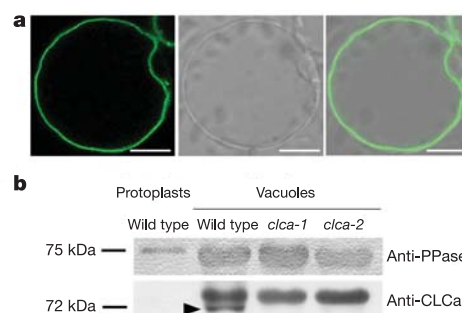


Figure 1 | AtCLCa resides in the tonoplast. **a**, Transient expression of AtCLCa–GFP fusion proteins in protoplasts. Laser-scanning images of GFP fluorescence (left), transmitted light (centre) and merge of both (right); scale bars correspond to 16 μm . **b**, Western blot analysis of protoplast and vacuolar membranes from wild-type, *clca-1* (ref. 9) and *clca-2* plants showed an amplification of the signal revealed by anti-pyrophosphatase (anti-PPase) IgG, indicating enrichment in the vacuolar fraction. An anti-CLCa IgG recognized AtCLCa (arrowhead in the blot) in the wild-type but not in the mutant vacuolar fractions, confirming the absence of AtCLCa protein in the mutants.

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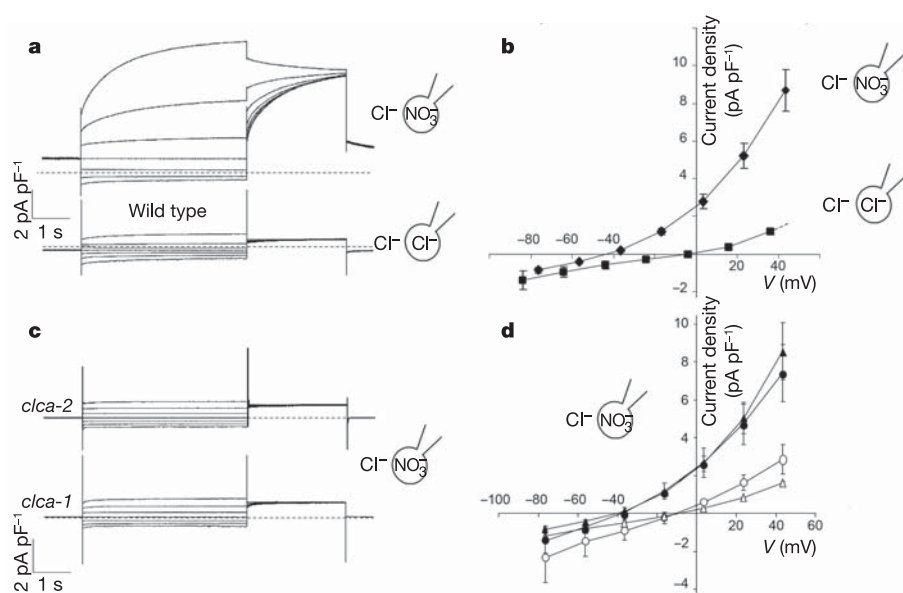


Figure 2 | Whole-vacuole NO_3^- currents are abolished in vacuoles from *clca* knockout mutants. **a**, Nitrate currents in wild-type vacuoles. Upper traces show results for vacuolar solution of 200 mM NO_3^- ; lower traces for vacuolar solution with 200 mM Cl^- . **b**, Steady-state I - V curves of wild-type vacuoles show selectivity for vacuolar NO_3^- (diamonds; $n = 10$) over Cl^- (squares; $n = 3$). **c**, Currents in mutant vacuoles with 200 mM vacuolar NO_3^- .

d, Steady-state I - V curves of wild-type (filled symbols) and mutant (open symbols) currents (*clca-2*, circles, $n = 19$ from 9 plants and corresponding wild-type control plants, $n = 3$ from 3 plants; *clca-1*, triangles, $n = 9$ from 3 plants and corresponding controls, $n = 5$ from 3 plants). **a**-**d**, Reference solutions used (see Methods). Error bars indicate s.e.m.

AtCLCa, we analysed vacuoles from the two independent *clca* knockout plants with the patch-clamp technique in the whole-vacuole configuration. To ensure that the different genotypes were at the same physiological and developmental stage, mutant and wild-type plants were first germinated *in vitro*, individually transferred to pots,

grown in identical controlled conditions and vacuoles of wild-type and knockouts were analysed in parallel on the same day. Figure 2c, d shows the currents recorded from *clca-2* (19 vacuoles, 9 plants) and *clca-1* (9 vacuoles, 3 plants) vacuoles. Notably, none of the mutant vacuoles analysed from these two independent knockouts showed a

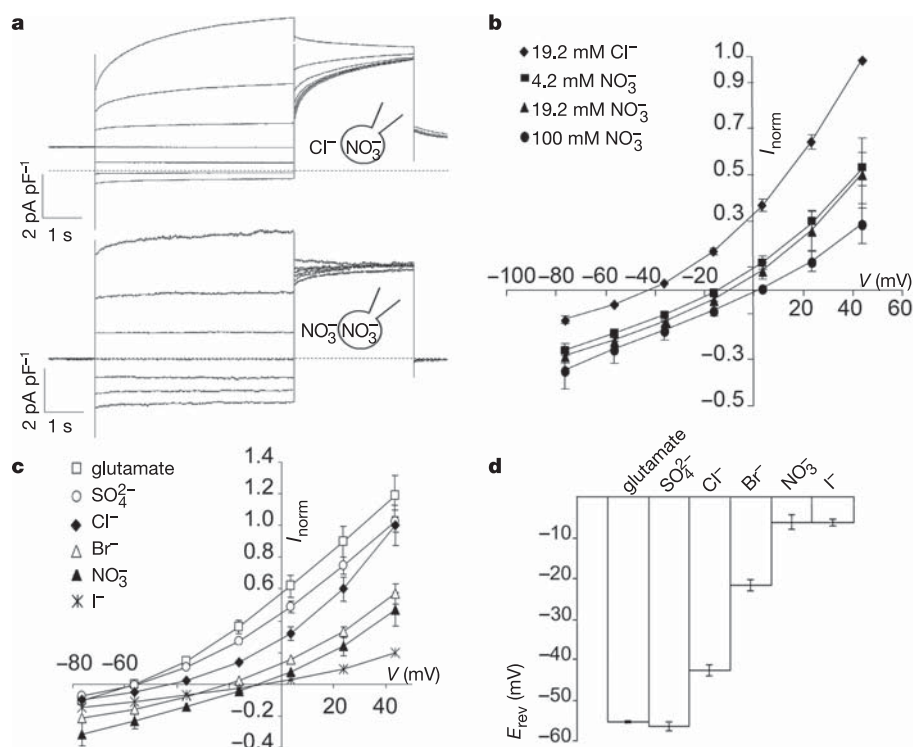


Figure 3 | AtCLCa mediates specific NO_3^- transport into the vacuole. **a**, Currents recorded from the same vacuole in reference solutions (upper traces) and after cytosolic perfusion with 19.2 mM NO_3^- (lower traces). **b**, Steady-state I - V curves in cytosolic 19.2 mM Cl^- and different cytosolic NO_3^- concentrations show that NO_3^- is transported into the vacuole

(by negative currents) at physiological (slightly negative) membrane potentials. **c**, Steady-state I - V curves with cytosolic solutions containing different anions (glutamate, SO_4^{2-} , Cl^- , Br^- , NO_3^- , I^-). **d**, Reversal potentials derived from I - V curves in **c**. **a**-**d**, A vacuolar reference solution was used (see Methods). Error bars indicate s.e.m.

current similar to the wild-type current. At 43 mV, the total current density was reduced by more than 60% in vacuoles of *clca-2* plants (control, $7.4 \pm 1.5 \text{ pA pF}^{-1}$; *clca-2*, $2.8 \pm 0.8 \text{ pA pF}^{-1}$) and by about 80% in vacuoles of *clca-1* plants (control, $8.6 \pm 1.5 \text{ pA pF}^{-1}$; *clca-1*, $1.6 \pm 0.1 \text{ pA pF}^{-1}$). The time-dependent part was decreased by 85% in *clca-2* and by more than 90% in *clca-1*. The discrepancy between time-dependent versus total current reduction in mutant vacuoles might reflect a minor contamination of the total current by instantaneous leak. In both mutant alleles, reversal potentials were different from wild type (Fig. 2d), with $E_{\text{rev}} = -16 \pm 2 \text{ mV}$ (*clca-2*) and $-11 \pm 2 \text{ mV}$ (*clca-1*), in agreement with the absence of a transport system in mutant vacuoles that would impose its equilibrium potential. Results on both alleles were fully concordant, and showed that the time-dependent part of the nitrate-selective current is missing and the residual current is less NO_3^- -selective in *clca-1* and *clca-2* mutants. In contrast, no marked difference in vacuolar malate currents^{15–18} could be detected between wild-type and the *clca-2* mutant plants (Supplementary Fig. 1). Taken together, these results allowed us to conclude that *AtCLCa* encodes the transport system mediating the novel nitrate current described above.

The main phenotype of the *clca* mutants is impaired nitrate storage in the vacuole. Therefore, we tested whether the current mediated by *AtCLCa* was able to transport nitrate from the cytosol to the vacuolar lumen. Wild-type plants were studied in whole-vacuole patch-clamp experiments, using solutions where cytosolic Cl^- was entirely substituted with NO_3^- . Figure 3a shows that cytosolic NO_3^- affected several characteristics of *AtCLCa* currents: induction of a shift of E_{rev} , and changes in current amplitudes and activation kinetics. The positive current (NO_3^- entering the cytosol) decreased

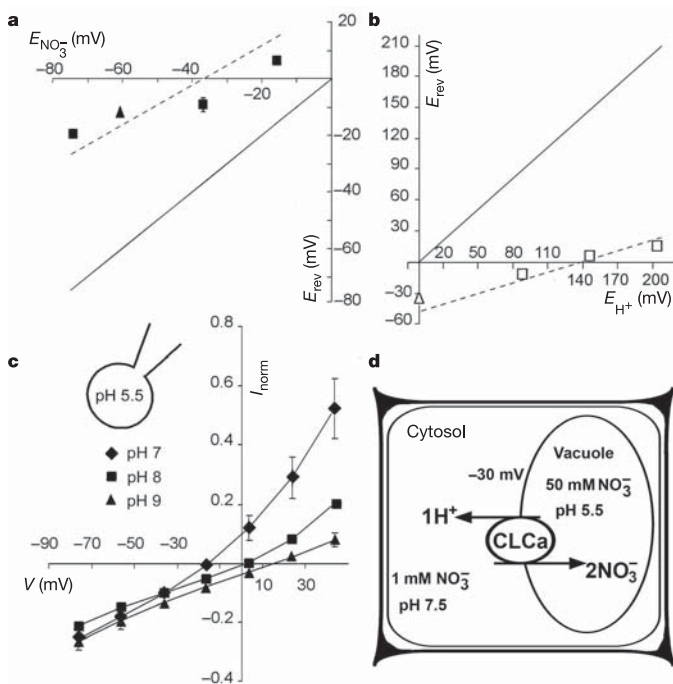


Figure 4 | *AtCLCa* functions as a $2\text{NO}_3^-/1\text{H}^+$ antiporter. **a, b**, E_{rev} varies with NO_3^- and pH gradients; plots of reversal potentials versus Nernst potentials for NO_3^- and H^+ are shown. **a**, Squares (from left), cytosolic NO_3^- 4.2, 19.2 and 100 mM, pH 7, all with reference solution of vacuolar 200 mM NO_3^- , pH 5.5; triangle, cytosolic 4.2 mM NO_3^- , pH 7, and vacuolar 100 mM NO_3^- , pH 5.5. **b**, Squares (from left), cytosolic pH 7, 8 and 9 with 4.2 mM NO_3^- , all with 200 mM NO_3^- , pH 5.5, vacuolar reference solution; triangle, vacuolar 200 mM NO_3^- , pH 7, and cytosolic 4.2 mM NO_3^- , pH 7. Dotted and continuous lines represent best fits obtained with transporter⁴ and Nernst equations, respectively. **c**, Steady-state I - V curves varying cytosolic pH as in **b**. **d**, Representation of the *AtCLCa* NO_3^-/H^+ antiporter in a plant cell. Error bars indicate s.e.m.

when nitrate was present in the cytosol, whereas the negative current (NO_3^- leaving the cytosol) increased. Figure 3b shows I - V curves obtained in different cytosolic NO_3^- concentrations, normalized with respect to bi-ionic conditions (see Supplementary Fig. 2 for current density I - V curves). The shift of E_{rev} to more positive values and the increase of the inward current indicated that *AtCLCa* is able to mediate NO_3^- influx into the vacuole. In 4.2, 19.2 or 100 mM NO_3^- and between -76 and -20 mV , the conductance of the transport system is constant (70 pS pF^{-1}), indicating a saturation of *AtCLCa* at low nitrate concentrations and physiological tonoplast potentials. To determine the selectivity sequence for the *AtCLCa* current, E_{rev} values (Fig. 3c, d) were measured in solutions where cytosolic Cl^- was entirely substituted by different anions. The following selectivity series was deduced from reversal potentials¹⁹: NO_3^- ($-6.0 \pm 2.0 \text{ mV}$) $\approx \text{I}^-$ ($-6.0 \pm 0.5 \text{ mV}$) $> \text{Br}^-$ ($-21.6 \pm 1.4 \text{ mV}$) $> \text{Cl}^-$ ($-43.3 \pm 1.4 \text{ mV}$) $> \text{SO}_4^{2-}$ ($-55.4 \pm 0.4 \text{ mV}$) $> \text{glutamate}$ ($-56.0 \pm 1.0 \text{ mV}$). The high selectivity for nitrate on the cytosolic side is in agreement with the nitrate-specific phenotype of *clca* knockout mutants.

Our experiments show that, at physiological membrane potentials (-40 to -20 mV ; Fig. 3b), *AtCLCa*-mediated current is able to transport NO_3^- into the vacuole (negative current), when cytosolic $[\text{NO}_3^-]$ is in a physiological range (4.2–19.2 mM). Nevertheless, there is a clear discrepancy between the measured E_{rev} and the Nernst potential for NO_3^- (Fig. 4a). This observation demonstrates that other ions are transported by *AtCLCa*. Previous studies^{4–6} showed that some members of the *CLC* family actually function as Cl^-/H^+ antiporters. To investigate this possibility, we monitored reversal potentials of *AtCLCa* current when cytosolic NO_3^- was changed in a fixed pH gradient (Fig. 4a) or when cytosolic pH varied in a fixed NO_3^- gradient (Fig. 4b). We observed that E_{rev} and current amplitudes varied when external pH was changed (Fig. 4b, c). To test whether an exchanger model accounts for these data, we used the antiporter equation^{4,20} (see Supplementary Information) to fit the plots in Fig. 4a, b. The fits gave a slope per decade of 40 mV and 20 mV, respectively (well below the nernstian slope of 58 mV per decade), and a stoichiometric ratio (r) of 0.44 and 0.53, respectively, meaning that two anions are transported for one proton. The sum of the two slopes was 60 mV, a value, within errors, identical to the Nernst slope per decade as required by the exchanger equation. When vacuolar NO_3^- and pH were varied, the observed reversal potentials were in accordance with values predicted by the exchanger model (Fig. 4a, b). These data led to the conclusion that *AtCLCa* functions as a $2\text{NO}_3^-/1\text{H}^+$ antiporter, which is in agreement with the $2\text{Cl}^-/1\text{H}^+$ ratio reported for *ClCec-1* (ref. 4).

In *Escherichia coli*, glutamates E148 and E203 are critical for Cl^-/H^+ coupling^{4–6}, and E203 is a hallmark that distinguishes *CLC* antiporters from channels²¹. Protein sequence alignment (Supplementary Fig. 3) shows that both glutamate residues are conserved in the *AtCLCa* sequence. This is in agreement with our results showing that *AtCLCa* functions as a NO_3^-/H^+ antiporter.

Our results unveil the molecular basis for nitrate accumulation in plant vacuoles. Indeed, the antiporter mechanism is a prerequisite for *AtCLCa*-mediated vacuolar nitrate accumulation: at a physiological tonoplast potential of -30 mV (ref. 22), a nitrate-selective channel would drive accumulation by a factor of 3, whereas a $2\text{NO}_3^-/1\text{H}^+$ exchange mechanism with a gradient of 2 pH units (vacuolar pH 5.5, cytosolic pH 7.5) allows an accumulation factor of 50, which is in agreement with reported values²² (Fig. 4d).

In the present work, we provide a demonstration of anion transport activity of a plant *CLC* protein. The high selectivity of *AtCLCa* for nitrate ions compared with chloride ions seems to be a peculiarity of this plant *CLC* and is in agreement with the *clca* knockout mutant phenotype. Finally, the present findings illustrate the exchanger mechanism of a *CLC* protein in the native membrane system and answer the biological question²³ regarding *CLC* antiporter function in an intracellular organelle.

METHODS

Localization. AtCLC-GFP carboxy-terminal fusions were obtained by cloning AtCLC α cDNA into the pA7-GFP plasmid (provided by K. Czempinski) and introduced into *Arabidopsis* cell suspension²⁴ protoplasts by PEG-mediated transformation. Confocal laser-scanning microscope observations were performed 24 h after transformation.

Membrane isolation and western blot analysis. Vacuolar suspensions were prepared²⁵ from mesophyll protoplasts of wild-type, *clca-1* (ref. 9) and *clca-2* (INRA collection, FST 171A06, T-DNA insertion in the fourth exon at 3,816 base pairs from the start codon) plants. Identical amounts of proteins (5 μ g protein per lane) were resolved by SDS–polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane (Hybond-cExtra, Amersham-Biosciences) and probed with rabbit antibody (Eurogentec) raised against the C terminus of AtCLC α (PHLDKHKSGKA) or *Arabidopsis* pyrophosphatase²⁶.

Electrophysiology. Currents were evoked in response to 5-s pulses from -77 to $+43$ mV in $+20$ -mV increments, followed by a 3-s tail pulse at -67 mV with a holding potential of -17 mV (after liquid junction potential correction²⁷). Bis-Tris-Propane (BTP) was chosen as an impermeable cation in order to minimise cation currents^{16,17}. Reference solutions contained: (cytosolic) 7.5 mM BTP, 15 mM HCl, 0.1 mM CaCl₂, 2 mM MgCl₂, 15 mM MES, pH 7, $\pi = 640$ mOsm adjusted with sorbitol; (vacuolar) 100 mM BTP, 200 mM HNO₃, 1 mM CaCl₂, 5 mM MgCl₂, 5 mM MES, pH 5.5, $\pi = 590$ mOsm with sorbitol. Nitrate cytosolic solutions in Figs 3 and 4 contained: (for 4.2 mM NO₃⁻) 0.1 mM Ca(NO₃)₂, 2 mM Mg(NO₃)₂, 15 mM MES, pH 7, 8 or 9 adjusted with BTP; (for 19.2 mM NO₃⁻ and 100 mM NO₃⁻ at pH 7) 15 mM HNO₃ and 7.5 mM BTP or 94.8 mM HNO₃ and 50 mM BTP were added, respectively. For selectivity analysis, Fig. 3c, d Cl⁻ was entirely substituted by the anion of interest in the cytosolic reference solution (see Supplementary Information). Experiments were designed to start in reference solutions and then switched to measuring cytosolic solution. In Figs 3b, c and 4c, currents were normalized to current at $+43$ mV in reference solutions. Nernst equilibrium potentials were calculated using ionic activities (Debye–Hückel equation²⁸). Error bars indicate s.e.m.

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Signal sequence directs localized secretion of bacterial surface proteins

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All living cells require specific mechanisms that target proteins to the cell surface. In eukaryotes, the first part of this process involves recognition in the endoplasmic reticulum of amino-terminal signal sequences and translocation through Sec translocons, whereas subsequent targeting to different surface locations is promoted by internal sorting signals¹. In bacteria, N-terminal signal sequences promote translocation across the cytoplasmic membrane, which surrounds the entire cell, but some proteins are nevertheless secreted in one part of the cell by poorly understood mechanisms^{2,3}. Here we analyse localized secretion in the Gram-positive pathogen *Streptococcus pyogenes*, and show that the signal sequences of two surface proteins, M protein and protein F (PrtF), direct secretion to different subcellular regions. The signal sequence of M protein promotes secretion at the division septum, whereas that of PrtF preferentially promotes secretion at the old pole. Our work therefore shows that a signal sequence may contain information that directs the secretion of a protein to one subcellular region, in addition to its classical role in promoting secretion. This finding identifies a new level of complexity in protein translocation and emphasizes the potential of bacterial systems for the analysis of fundamental cell-biological problems⁴.

S. pyogenes (group A streptococcus) is best known as the cause of acute pharyngitis ('strep throat') but also causes severe diseases that result in more than 500,000 deaths each year⁵. The M protein, which is the most extensively studied virulence factor of *S. pyogenes*, is a fibrillar protein that inhibits phagocytosis^{6–8}. Classical studies showed that M protein is secreted at the septum, where it becomes associated with new cell wall, which is formed in this region^{9,10}. The newly secreted M protein, from which the signal sequence has been cleaved off, does not remain associated with the cytoplasmic membrane but is covalently attached to the rigid peptidoglycan layer⁶ and is subsequently distributed over the entire bacterial surface together with the growing peptidoglycan^{9,10}. However, all secreted *S. pyogenes* proteins are not translocated at the septum, as shown by recent studies of the extracellular protease SpeB, which is secreted at a single asymmetrically located site^{2,11}.

The possibility that M protein is secreted at the septum by default has not been excluded, because translocation of this protein may occur most easily where new wall is formed; however, we proposed that a specific mechanism promotes the secretion of M protein at the septum. This hypothesis implied that some surface proteins might be secreted at other sites, a situation that would allow comparative analysis. Because the fibronectin-binding PrtF is mainly located at the old poles of growing *S. pyogenes*¹², we analysed whether PrtF and M protein are secreted in different regions. For this purpose we treated a strain of serotype M6 with trypsin, which removes M protein and PrtF without killing the bacteria¹⁰ (M.S.-C., F.C. & G.L.,

unpublished observations), and used immunofluorescence microscopy to analyse where new protein appeared. In agreement with early studies¹⁰, M6 was distributed over the entire surface of untreated bacteria, was absent from trypsinized bacteria, and reappeared at the septum (Fig. 1a, left). A similar analysis showed that PrtF was located mainly at the old poles of untreated bacteria, was absent from trypsinized bacteria, and preferentially reappeared at the poles (Fig. 1a, right). Thus, M6 and PrtF are largely secreted in different regions. No signals were observed when mutants lacking M6 or PrtF were subjected to a similar analysis, demonstrating that the detection systems were specific (data not shown).

To analyse secretion by a different technique and allow quantitative analysis we employed immunogold electron microscopy to reveal proteins reappearing after trypsinization. Each daughter bacterium was divided into three zones (Fig. 1b) and gold particles in each zone were counted for a large number of cells (Fig. 1c). In agreement with the immunofluorescence microscopy analysis, most of the reappearing M6 was located in the zone including the septum, whereas reappearing PrtF was located mainly in the zone including the old pole, although almost 50% of the new PrtF was found in the other two zones, in particular at the septum. The results were statistically highly significant, focusing interest on the molecular basis for the difference.

The sequences of M protein and PrtF vary between strains, but not within a strain. We proposed that the localized secretion of these proteins is governed by linear sequences conserved within each protein species. For M protein, this hypothesis focused interest on three conserved regions: the N-terminal signal sequence¹³, an internal 22-residue region designated 22R (ref. 14) and the carboxy-terminal region containing the LPXTG motif, which is required for sortase-mediated anchoring of surface proteins to the cell wall envelope^{15,16} (Fig. 2a). Because both M protein and PrtF harbour LPXTG-motif sorting signals^{6,17}, it seemed unlikely that this region would also be implicated in directing localized secretion. A function for the 22R region was ruled out by the finding that a mutant lacking this region was still secreted at the septum (data not shown). This situation indicated a possible role for the signal sequence in M protein, and also that in PrtF, in localized secretion. The signal sequences of these proteins are unrelated but highly conserved within each protein species, and they are relatively long^{18,19}, as commonly seen in Gram-positive bacteria²⁰ (Fig. 2b).

The role of the signal sequences in directing localized secretion was analysed with domain swaps in which the secretion of PrtF was directed by the signal sequence of M6, and vice versa. For the construction of these domain swaps, it was essential to know the exact location of the signal peptidase cleavage sites in M6 and PrtF. For M6 this site was known¹⁸, and for PrtF it was determined by N-terminal sequencing of the partly purified protein extracted from

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streptococci (Supplementary Fig. 1S). On the basis of this information we constructed domain swaps. To avoid possible problems during processing by signal peptidase in *S. pyogenes*, each construct included some amino-acid residues from the mature part of the protein contributing the signal sequence (Fig. 2b). In particular, the construct containing the signal sequence of M6 included ten residues from the mature part because the inclusion of fewer residues caused toxicity problems during cloning. The limited length of this region and its extreme sequence variability among M proteins (Supplementary Fig. 2S) make it very unlikely that it has a function in determining the intracellular secretion site. A shuttle plasmid was used to transfer the two parental genes and the genes encoding domain swaps into *S. pyogenes* strain SAM2, which lacks the expression of M6 and PrtF. The two domain swaps are designated MF and FM (Fig. 2a), and the plasmid-carrying strains are referred to as pM6, pPrtF, pMF and pFM.

To analyse whether the domain swaps promoted the expression of the corresponding mature proteins on the surface of *S. pyogenes*, the four plasmid-carrying strains were analysed for their ability to bind anti-M serum and radiolabelled fibronectin (Fig. 2c). Anti-M serum reacted equally well with the pM6 and pFM strains, and fibronectin showed similar binding to the two strains pPrtF and pMF. Thus, M protein and PrtF were expressed on the bacterial surface, whether encoded by wild-type genes or by genes encoding domain swaps. To analyse whether the domain swaps were properly processed, surface proteins were extracted and after partial purification the relevant proteins were subjected to N-terminal sequencing (Supplementary Fig. 1S). The domain swaps were processed at the expected sites (Fig. 2b), implying that they could be used to analyse the role of signal sequences for the secretion patterns of M6 and PrtF.

The secretion of the four plasmid-encoded proteins was analysed by quantitative immunogold electron microscopy. The wild-type M6

protein was evenly distributed over the surface of untrypsinized bacteria, whereas PrtF was preferentially located at the old pole (Fig. 3a). After trypsinization, M6 reappeared at the septum, whereas PrtF was preferentially secreted at the old pole (Fig. 3b). Although PrtF reappeared not only at the pole but also at the septum, the difference from M6 was statistically highly significant. Thus, the secretion patterns of plasmid-encoded M6 and PrtF were very similar to those observed with chromosomally encoded proteins (Fig. 1c). Analysis of the two domain swaps demonstrated that they had properties similar to those of the wild-type protein from which the signal sequence was derived (Fig. 3c, d). On untrypsinized bacteria (Fig. 3c), PrtF was evenly distributed over the bacterial surface when secretion was governed by the M6 signal sequence in the MF construct. Moreover, M6 was found mainly at the pole of untrypsinized bacteria when its secretion was directed by the PrtF signal sequence in the FM construct (Fig. 3c). The regions where the proteins were secreted after trypsinization were also determined by the signal sequences (Fig. 3d). The MF domain swap caused most PrtF to reappear at the septum, whereas the FM domain swap caused most M6 to be secreted in the zone including the old pole. Similar results were obtained by immunofluorescence microscopy analysis (data not shown). Thus, the signal sequences of M6 and PrtF direct secretion to distinct subcellular regions. This result cannot be explained by the secretion of M protein and PrtF at uniformly distributed sites, followed by diffusion in the membrane and capture at different sites²¹, because M protein and PrtF are not membrane proteins but are entirely translocated through the cytoplasmic membrane, implying that their signal sequences direct localized secretion before processing.

The signal sequence of M6, but not that of PrtF, contains a YSIRK-like motif, which has been implicated in optimal secretion^{22,23}. To analyse whether this motif (YSLRK in M proteins) influences secretion at the septum, we used site-specific mutagenesis in the

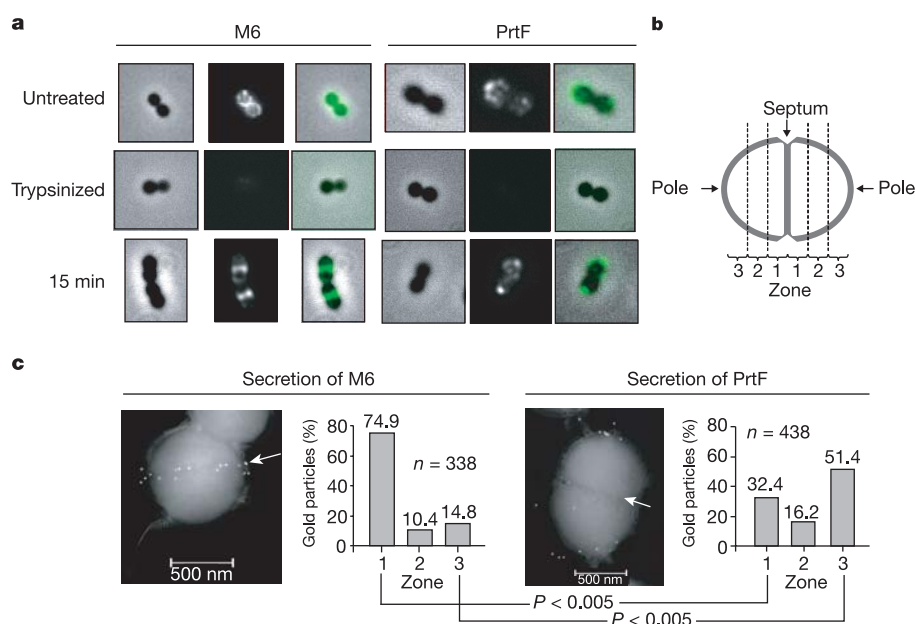


Figure 1 | Surface distribution and secretion pattern for M6 and PrtF in *S. pyogenes*. **a**, Strain JRS4 analysed by immunofluorescence microscopy before trypsinization, immediately after trypsinization, and after growth for 15 min in the absence of trypsin. Each horizontal row shows the same cells as seen by phase contrast (left), immunofluorescence microscopy (middle) and merged (right, with immunofluorescence microscopy pseudocoloured as green). M6 was detected with anti-M serum; PrtF was detected with a fibronectin fragment, followed by anti-fibronectin serum. **b**, For electron microscopy analysis, the surface of each daughter bacterium was divided into three zones by dividing the distance between the youngest detectable septation and the pole by three. **c**, Reappearance of M6 and PrtF 15 min after

trypsin treatment, analysed by immunogold electron microscopy of JRS4-derivatives SAM1 and JRS145. Gold particles in each zone were counted for many bacteria and are shown as percentages of the total number of particles (n) in the three zones. Representative electron micrographs are included with the septum indicated (arrow). Some gold particles seem not to be in direct contact with the bacteria; this is a result of cell shrinking during sample preparation. The fraction of M6 reappearing in zone 1, in comparison with the fraction of PrtF reappearing in this zone, was highly significant. Similarly, the preferential secretion of PrtF in zone 3 was highly significant.

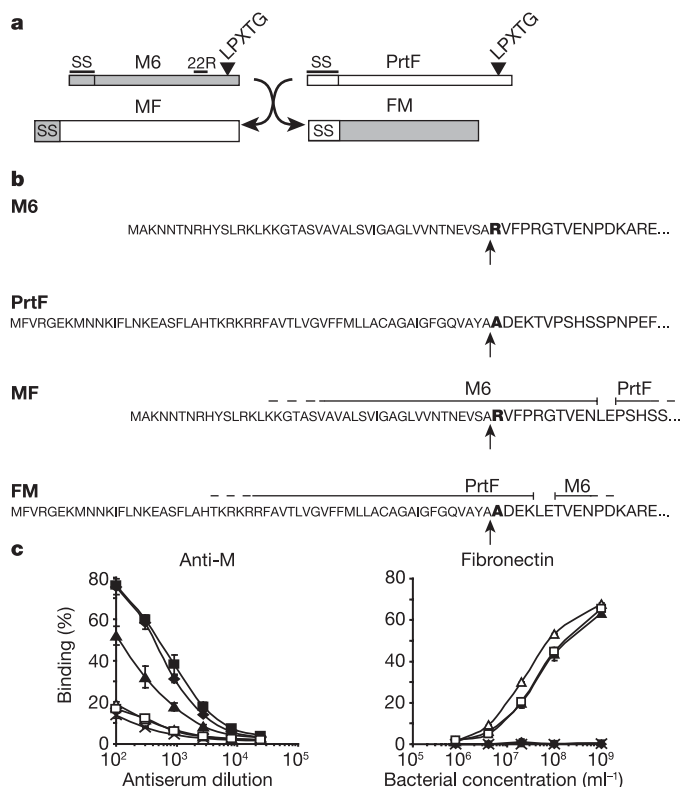


Figure 2 | Domain swaps derived from M6 and PrtF are expressed on the streptococcal surface. **a**, Schematic representation of M6 and PrtF and the domain swaps MF and FM. The position in M6 of three conserved regions is indicated: the signal sequence (SS), the 22R region and the LPXTG motif. **b**, Signal sequences and signal peptidase cleavage sites (arrows). In the domain swaps, the cloning procedure caused the introduction of the sequence LE between the parts derived from M6 and PrtF. **c**, Surface expression in *S. pyogenes* of the proteins shown in **a**, encoded on a plasmid (strains pM6 (filled squares), pPrtF (open triangles), pMF (open squares) and pFM (diamonds)). Expression of M6 and PrtF was detected with anti-M serum and ¹²⁵I-labelled fibronectin, respectively. Strains JRS4 (filled triangles) and SAM2 (crosses) were used as positive and negative controls, respectively. Values are means $\pm$ s.d. for three experiments.

M5 system to construct strains expressing proteins with the sequence YAARK or YSAAK in the signal sequence. Surface expression of M protein was not affected in the mutant strains, confirming that the YSLRK motif is not essential for secretion²³ (Supplementary Fig. 3S). Immunogold electron microscopy analysis showed that M protein was secreted at the septum not only in the wild-type control but also in the two mutants (Supplementary Fig. 3S). The YSLRK motif is therefore probably not required for localized secretion of M protein. We propose that a different region within the signal sequence of M protein promotes binding to the cell division complex, either directly or through a cognate adaptor, thereby promoting secretion in the equatorial region. It is less easy to envisage how PrtF is secreted in regions not located at the septum, but PrtF may be secreted at specialized sites, as described for secreted proteins of *Bacillus subtilis*²⁴ and *Listeria monocytogenes*²⁵. The dynamics of cell wall growth may also contribute to the preferential localization of PrtF to the old pole²⁵. The signal sequence of PrtF contains a twin-arginine (RR) sequence, indicating that it might be translocated through Tat translocons²⁶, but this is unlikely because *S. pyogenes* seems to lack a Tat system²⁷ and because PrtF lacks the RR-containing consensus motif²⁶.

Studies of SpeB recently led to the suggestion that not only this protease but all *S. pyogenes* proteins are secreted at the single ExPortal site, which was reported to contain all SecA (ref. 2). However, our data and those of Swanson *et al.*¹⁰ indicate that proteins are secreted at multiple sites. We therefore determined the distribution of SecA in wild-type bacteria, using immunogold electron microscopy of thin sections. The analysis employed antiserum against SecA from *B. subtilis*, which recognizes SecA from *S. pyogenes*² (Fig. 4a). In this analysis, SecA was found throughout the periphery of *S. pyogenes* and also in the cytoplasm, and no evidence was obtained for exclusive localization to a single site (Fig. 4b). Similar results were obtained with bacteria grown under the conditions used to study the ExPortal site² (Supplementary Fig. 4S). These data indicate that the Sec machinery might be circumferentially distributed in *S. pyogenes*, in agreement with studies of other bacteria^{24,28}.

Thus, our data identify a new function for signal sequences, the ability to direct localized secretion. Because this function might be important for bacterial virulence, the components involved are of potential interest as targets for antimicrobial therapy.

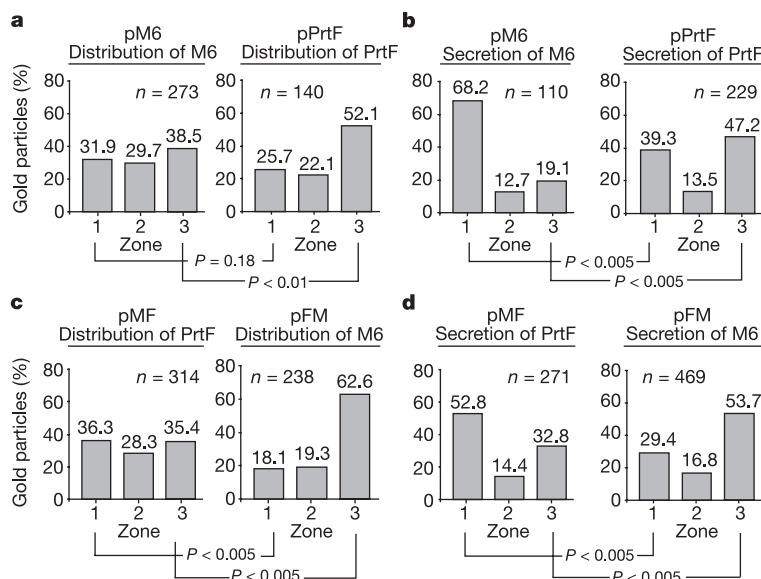


Figure 3 | Signal sequences direct secretion to different subcellular regions. **a–d**, Surface distribution and secretion sites for M6 and PrtF and the domain swaps MF and FM, analysed by quantitative immunogold electron microscopy. The strains used are described in Fig. 2. Panels **a** and **c**

show distributions on untreated bacteria, whereas **b** and **d** show the location of new protein appearing 15 min after trypsinization. Statistical significance is indicated.

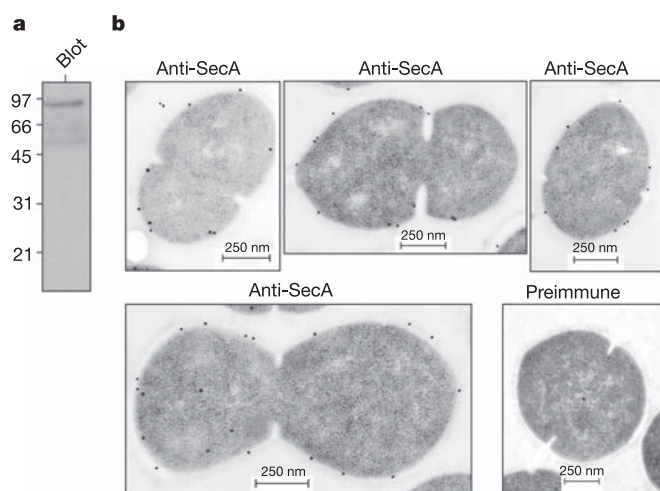


Figure 4 | Distribution of SecA in *S. pyogenes*. **a**, Western blot showing that antiserum against *B. subtilis* SecA recognizes a protein of the expected size in a lysate of *S. pyogenes*. The numbers at the left show the positions of molecular mass markers (in kDa). **b**, SecA is distributed over the entire cell periphery in *S. pyogenes*. Thin sections of strain JRS4 were incubated with anti-SecA or preimmune serum as indicated, and bound antibodies were detected by immunogold electron microscopy. Scale bars, 250 nm.

METHODS

Bacterial strains. The wild-type *S. pyogenes* strain JRS4 expresses M6 and PrtF, JRS145 is an M6-negative mutant, SAM1 is a PrtF-negative mutant, and SAM2 lacks both M6 and PrtF (ref. 12). Strains of *S. pyogenes* were constructed in which the parental proteins M6 and PrtF, and two domain swaps derived from these proteins, were encoded by genes carried by plasmid pLZ12Spec (Supplementary Methods).

Immunofluorescence and immunogold electron microscopy. The distribution of surface proteins on untrypsinized *S. pyogenes* was analysed for bacteria grown to late exponential phase. The site of secretion of new protein was analysed in trypsinized bacteria incubated at 37°C for 15 min, to allow protein synthesis. The methods used to detect M6 and PrtF employed rabbit antibodies, as described in Supplementary Methods.

Statistical analysis. For quantitative analysis of immunogold electron microscopy, we compared the fraction of gold particles in zone 1 (or zone 3) for M6 and PrtF, tested for equality of two binomial distributions (for example zone 1/zones 2 and 3) approximated with a normal distribution and calculated the significance (*P*) of a 95% confidence interval for the differences (percentages) in gold staining between the zones, as indicated.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Mechanism limiting centrosome duplication to once per cell cycle

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The centrosome organizes the microtubule cytoskeleton and consists of a pair of centrioles surrounded by pericentriolar material. Cells begin the cell cycle with a single centrosome, which duplicates once before mitosis. During duplication, new centrioles grow orthogonally to existing ones and remain engaged (tightly opposed) with those centrioles until late mitosis or early G1 phase, when they become disengaged¹. The relationship between centriole engagement/disengagement and centriole duplication potential is not understood, and the mechanisms that control these processes are not known. Here we show that centriole disengagement requires the protease separase² at anaphase, and that this disengagement licences centriole duplication in the next cell cycle. We describe an *in vitro* system using *Xenopus* egg extract and purified centrioles in which both centriole disengagement and centriole growth occur. Centriole disengagement at anaphase is independent of mitotic exit and Cdk2/cyclin E activity, but requires the anaphase-promoting complex and separase. In contrast to disengagement, new centriole growth occurs in interphase, is dependent on Cdk2/cyclin E, and requires previously disengaged centrioles. This suggests that re-duplication of centrioles within a cell cycle is prevented by centriole engagement itself. We propose that the 'once-only' control of centrosome duplication is achieved by temporally separating licensing in anaphase from growth of new centrioles during S phase. The involvement of separase in both centriole disengagement and sister chromatid separation would prevent premature centriole disengagement before anaphase onset, which can lead to multipolar spindles and genomic instability^{3,4}.

Cells in the G1 phase of the cell cycle have a single centrosome containing two centrioles joined loosely by cohesion fibres⁵. At the G1/S transition, new centrioles grow orthogonally from the two pre-existing centrioles. The new centrioles elongate until late G2 and remain in a tightly opposed orthogonal configuration through early M phase¹. We will refer to tightly opposed centriole pairs as 'engaged centrioles'. In early mitosis the two centrosomes, each containing a pair of engaged centrioles, separate and participate in mitotic spindle pole formation. The engaged centriole pairs within each centrosome become disengaged at some point between the end of mitosis and early G1, losing their strict orthogonal configuration¹. We will refer to this process as 'centriole disengagement' (the term 'centriole disorientation' has been used previously). Centrosome duplication requires Cdk2 and its associated cyclins^{6–9}; however, the continued presence of Cdk2 activity during S phase arrest is not sufficient to promote a second round of centrosome duplication in the same cell cycle¹⁰. The presence of other mechanisms to regulate duplication was indicated by cell fusion experiments revealing a centrosome-intrinsic block to re-duplication during a single cell cycle¹⁰. The nature of this centrosome-intrinsic block, and how the block is relieved so that centrosomes can be duplicated in the next cell cycle, is unknown.

The three essential events in centrosome duplication are: centriole disengagement, centriole growth and centrosome separation. In most somatic cell types, disengaged centrioles remain joined by cohesion fibres during interphase, preventing centrosome separation; however, this cohesion is usually not present in the rapid embryonic divisions in many species^{11,12}, and disruption of centrosome cohesion in human cells has no effect on cell viability^{5,13}. In contrast, the engagement and disengagement states of centrioles during the cell cycle are conserved^{1,14} and correlated with their duplication potential¹⁵. This suggests a model in which centriole disengagement at the end of mitosis licences centrioles for duplication, and the engaged centrioles in S and G2 are not substrates for duplication¹⁵.

We developed an *in vitro* system using *Xenopus* egg extract and purified centrosomes to determine the regulation of centriole disengagement. We used antibodies against two centriolar proteins to distinguish engaged from disengaged centrioles (Supplementary Fig. 1). Each engaged centriole pair has two foci of centrin, a protein in the centriole distal lumen¹⁶, and one focus of C-Nap1, a protein at the free (not engaged) proximal end of centrioles¹³ (2:1 ratio of centrin:C-Nap1) (Supplementary Fig. 1a–c). Disengaged centrioles have one centrin focus and one C-Nap1 focus per centriole (1:1, centrin:C-Nap1). In centrosomes purified from S-phase HeLa cells for the assay described below, greater than 95% of the centrioles were in the engaged configuration (2:1, centrin:C-Nap1; Supplementary Fig. 1d). Although centriole engagement was preserved during purification, centrosome cohesion was often disrupted such that single centriole pairs were observed (Supplementary Fig. 1d).

Xenopus egg extract was used to characterize centriole disengagement activity. S-phase centrosomes with engaged centrioles were incubated with cycling extract. Most centrioles remained engaged through the first interphase and entry into mitosis (Fig. 1a, 60 min).

Disengagement was observed 20 min later when the extract exited mitosis and returned to interphase (Fig. 1a, 80 min). Similar results were obtained with cytosolic factor (CSF) extract released from arrest by calcium addition (CSF-released extract) (Fig. 1b, 40 min). These results show that disengagement activity in these extracts occurs at the mitosis/interphase transition, as observed *in vivo*¹. To confirm that centriole disengagement and separation occurred, centrosomes purified from cells expressing centrin–green fluorescent protein (GFP) were used. A difference in GFP fluorescence intensity between two centrioles of a pair was consistently observed, presumably reflecting a difference in centrin levels between mother and daughter centrioles. Centrin–GFP centrosomes were incubated in CSF-released extract, and examined by time-lapse microscopy. In each of ten centriole doublets observed the centrioles disengaged and separated from each other (Fig. 1c). The separated centrioles were able to recruit pericentriolar material (Fig. 1c, fixed) and nucleate microtubules (Fig. 1c).

In contrast, when engaged centrioles were incubated with either

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interphase-arrested extract or CSF-arrested extract no disengagement was observed (Fig. 1d). To confirm that this reflected a failure of disengagement rather than disengagement followed by new centriole growth, centrin–GFP centrosomes were used. In this case, any newly formed centrioles would be GFP-negative, because the extract lacks soluble centrin–GFP. Only doublets in which both centrioles were GFP-positive were observed (Fig. 1d), indicating lack of centriole disengagement and of new centriole growth from engaged centrioles.

The combination of CSF-released extract with centrin–GFP-labelled centrioles provided a rapid assay to probe the requirements for centriole disengagement. We tested whether disengagement requires Cdk2/cyclin E, a cell cycle kinase required for centrosome duplication^{6–9}. The Cdk2 inhibitor $\Delta 34$ Xic1 blocked mitotic entry (Fig. 2), as reported¹⁷, but had no effect on centriole disengagement (Fig. 2). It is important to distinguish the disengagement activity

described here from the previously reported Cdk2-dependent centriole separation activity in interphase *Xenopus* egg extract⁷. The previous work used G1 centrosomes with disengaged centrioles⁷, and the activity observed was probably the breakage of centrosome cohesion, which is distinct from centriole disengagement.

We next tested whether disengagement requires mitotic exit and entry into interphase. Non-degradable $\Delta 90$ cyclin B was used to block mitotic exit in CSF-released extract (anaphase extract^{18,19}). The extract remained in mitosis, as assayed by sperm chromatin morphology (Fig. 2), but centriole disengagement occurred by 40 min after calcium addition. Thus, centriole disengagement requires neither inactivation of Cdk1 activity nor an interphase-specific activity. Addition of calcium to a CSF extract results in activation of the anaphase-promoting complex (APC). We tested whether centriole disengagement requires APC by adding an APC peptide inhibitor¹⁸ to CSF extract before the addition of centrioles and calcium. APC inhibition blocked centriole disengagement, whereas a mutant control peptide had no effect (Fig. 2), indicating that APC is required for centriole disengagement.

A key target of APC activity is separase, a protease that cleaves the cohesin complex at anaphase to facilitate the separation of sister chromatids². Two pathways inhibit the activity of separase before anaphase: securin²⁰ and high levels of Cdk1/cyclin B¹⁹; both are released at anaphase by APC activation. We tested the involvement of separase in centriole disengagement in two ways. First, we used an excess of $\Delta 90$ cyclin B, which inhibits separase but not APC¹⁹. A twofold increase in added $\Delta 90$ cyclin B blocked centriole disengagement (Fig. 3a). This result indicates that APC is not directly responsible for disengagement, because it is still active in these extracts¹⁹, and is consistent with a requirement for separase. To test further the role of separase in centriole disengagement, a non-degradable form of *Xenopus* securin (Xsecurin^{dm}) was used to inhibit separase. Incubation of Xsecurin^{dm} with extract as previously described²⁰ efficiently blocked separase activity but otherwise allowed cell cycle progression (Fig. 3b). Both chromatid separation and centriole disengagement were blocked in Xsecurin^{dm}-treated extract (Fig. 3b), whereas both proceeded in mock-treated extract. Consistent with the failure of disengagement, the centriole doublets were contained within a single focus of pericentriolar material (Fig. 3b, fixed). This suggests that separase activation is required for centriole disengagement as well as sister chromatid separation, both of which occur at anaphase.

We used the centriole disengagement assay to examine the relationship between disengagement and centriole growth. Engaged centrioles were incubated in CSF-released extract as in Fig. 1b but the time in extract was increased to allow for centriole growth. We found

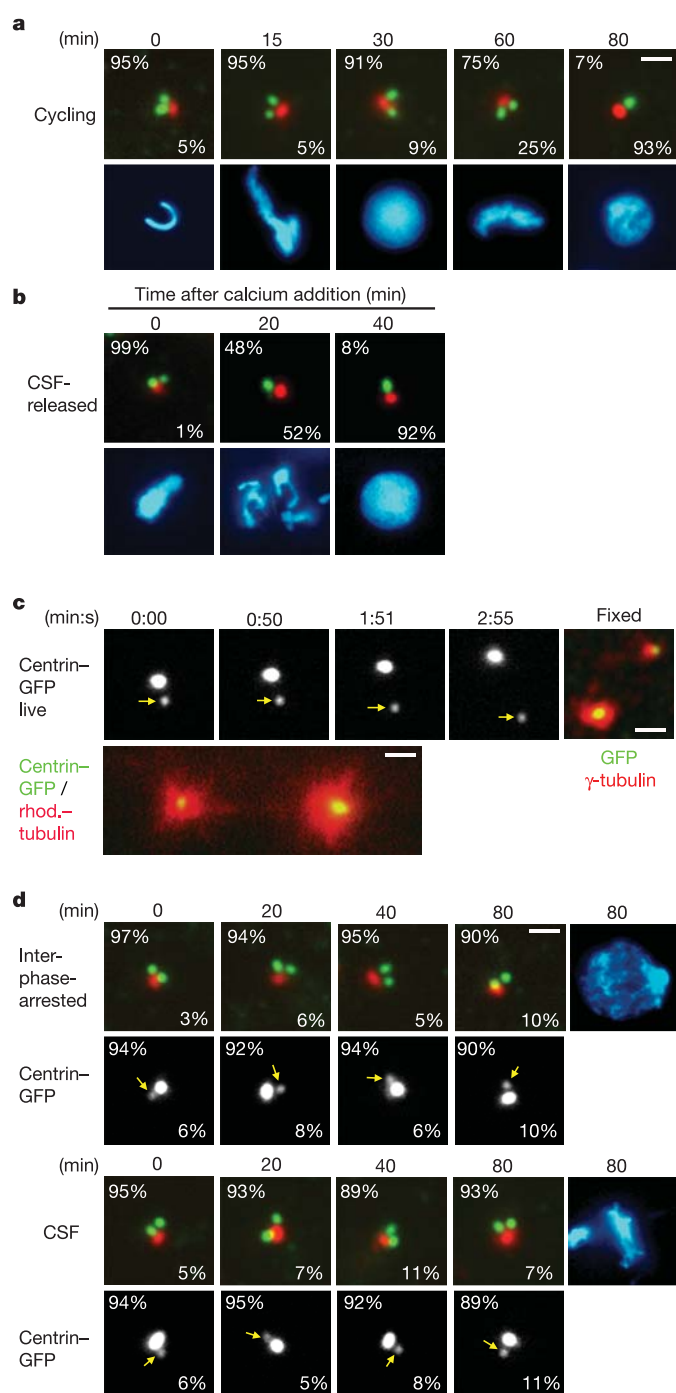


Figure 1 | Centriole disengagement activity is present in late mitosis.

a, b, d, S-phase centrosomes with engaged centrioles (non-labelled or centrin–GFP-labelled) and sperm nuclei were incubated with extract for the indicated times. At each time point, centriole configuration was determined ($n = 100$ centrosomes) with anti-centrin (green) and anti-C-Nap1 (red) antibodies. Anti-C-Nap1 antibodies react specifically with human C-Nap1 (not shown), thus only the input human centrosomes are labelled. Cell cycle stage was determined by the morphology of sperm DNA stained with Hoechst 33342 (blue). Centrin–GFP-labelled centrioles were visualized directly by fluorescent microscopy. The difference in centrin–GFP intensity between two centrioles of a pair was consistently observed, presumably reflecting a difference in centrin levels between mother and daughter centrioles (indicated with yellow arrows). The percentage of doublets is shown in the upper left of each panel; percentage of singlets in lower right. **c**, Time-lapse imaging of centriole disengagement and separation in CSF-released extract, visualized by centrin–GFP fluorescence. A sample of the reaction was also fixed and stained with anti-GFP (green) and anti- γ -tubulin (red) antibodies (fixed). Microtubule nucleation from disengaged centrioles in extract is shown with centrin–GFP (green) and rhodamine-labelled tubulin (red) as indicated (bottom panel). Scale bars: 1 μ m.

that centrioles became disengaged as above, followed by formation of new centrioles, so that at the subsequent prophase (90 min) most singlets had again become doublets (Fig. 4a). Inhibiting Cdk2/cyclin E with $\Delta 34\text{Xic1}$ blocked centriole growth but not centriole disengagement (Fig. 4a), indicating that growth and disengagement are separable activities.

We have shown that centriole disengagement activity is present at anaphase (Figs 1a, b, 2 and 3b), that centriole growth activity is present during interphase (Fig. 4a), and that centriole growth does not occur from engaged centrioles (Fig. 1a, c). This suggests that centriole growth can only occur from disengaged centrioles. To test this, engaged centrioles were allowed to disengage in CSF-released extract, then diluted tenfold into cycling extract. In contrast to the results with engaged centrioles (Fig. 1a, c), new daughter centrioles grew from these disengaged mothers during the first interphase (Fig. 4b). These newly duplicated centriole doublets became disengaged again at the following anaphase (Fig. 4b, 80 min). This result indicates that centriole disengagement is a prerequisite for centriole growth, and therefore that the engaged state of centrioles after duplication is a mechanism for the centrosome-intrinsic block to re-duplication.

We have described a mechanism that limits centrosome duplication to once per cell cycle. This control is achieved by separating centriole duplication into two distinct phases (Supplementary Fig. 2). First, centrioles are disengaged during anaphase—dependent on

separase activity—which licences them for duplication in the subsequent interphase. Second, the disengaged centrioles support the growth of new centrioles in interphase—dependent on Cdk2/cyclin E activity. Newly duplicated centrioles are engaged, and disengagement activity is absent in interphase, preventing a second round of

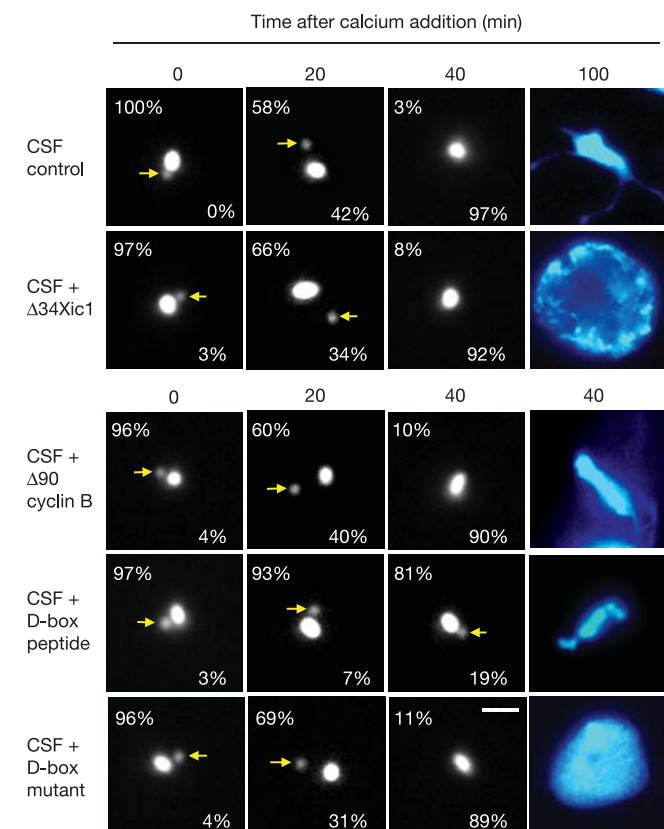


Figure 2 | Molecular requirements for centriole disengagement. Engaged S-phase centrioles purified from HeLa cells expressing centrin-GFP and sperm nuclei were incubated with CSF extracts pre-treated with reagents as indicated, and calcium was added to release CSF arrest (time after calcium addition is shown). DNA was stained with Hoechst 33342 (blue). Centrioles at each time point ($n = 100$) were visualized directly by fluorescence microscopy. Yellow arrows indicate daughter centrioles. The percentage of doublets is shown in the upper left of each panel; percentage of singlets in lower right. The final concentrations of proteins/peptides added in the extract are: 0.5 mg ml^{-1} $\Delta 34\text{Xic1}$; 0.04 mg ml^{-1} $\Delta 90$ cyclin B; 0.25 mg ml^{-1} D-box peptide; 0.25 mg ml^{-1} D-box mutant peptide. Scale bar: $1 \mu\text{m}$.

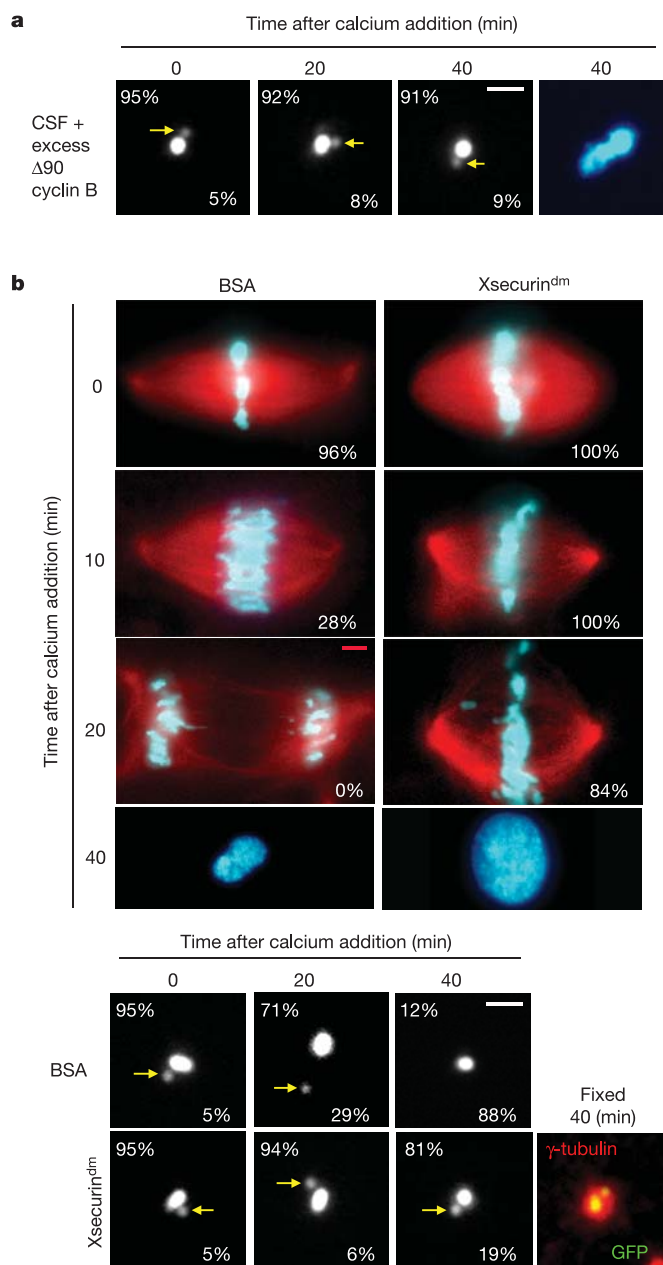


Figure 3 | Separase is essential for centriole disengagement. **a**, CSF extract was pre-treated with excess $\Delta 90$ cyclin B (0.08 mg ml^{-1}). S-phase centrosomes containing centrin-GFP-labelled centrioles were incubated and assayed as described in Fig. 2. Yellow arrows indicate daughter centrioles. **b**, Chromatid separation and centriole disengagement assays were performed in BSA-treated or Xsecurin^{dm}-treated extract. Images show the Hoechst-33342-stained chromosomes (blue) and rhodamine-labelled mitotic spindles (red) at times after metaphase release (min). The percentage of spindles at metaphase for each time point ($n = 25$ spindles) is indicated. In the centriole disengagement assay, centrin-GFP-labelled centrioles were used for direct visualization by fluorescence microscopy. Yellow arrows indicate daughter centrioles. A sample of the reaction was also fixed and stained with anti-GFP (green) and anti- γ -tubulin (red) antibodies (fixed, 40 min). The percentage of doublets is shown in the upper left of each panel; percentage of singlets in lower right. The white and red scale bars represent $1 \mu\text{m}$ and $10 \mu\text{m}$, respectively.

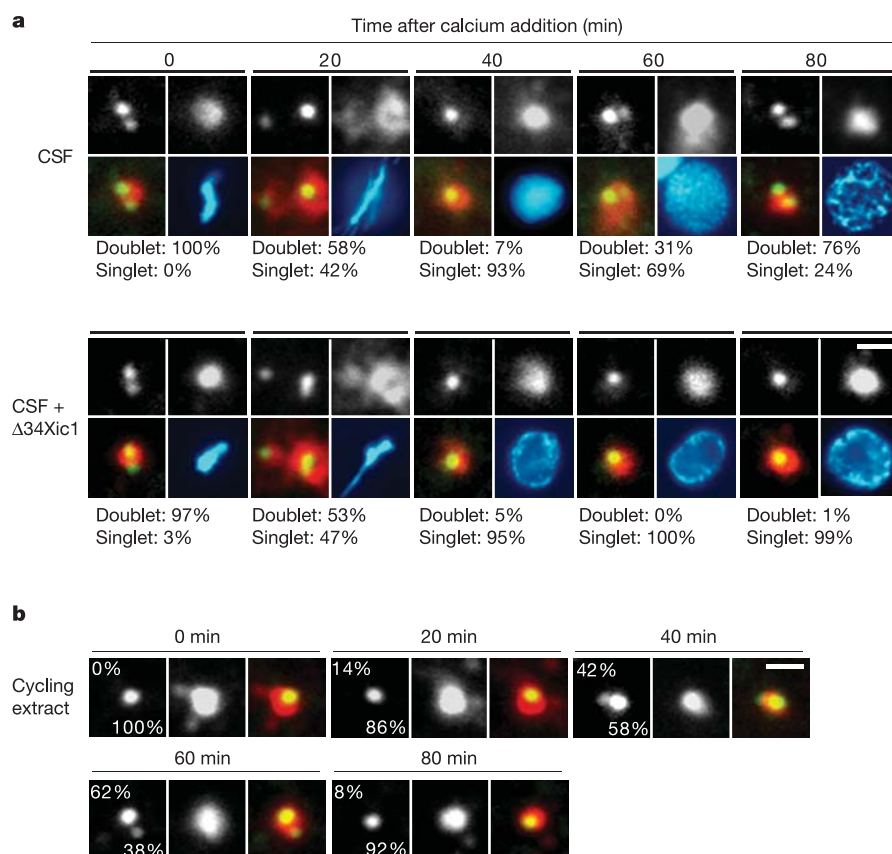


Figure 4 | Centriole disengagement is required for centriole growth.

a, Sperm nuclei and S-phase centrosomes containing engaged centrioles were incubated with CSF extracts pre-treated with buffer or $\Delta 34Xic1$ (0.5 mg ml^{-1}), and calcium was added to release CSF arrest (time after calcium addition is shown). DNA is stained with Hoechst 33342 (blue). Input human centrosomes were visualized by anti-centrin (upper left of each panel, green in merged image) and anti-human pericentrin (upper right, red in merged image) antibodies at the indicated time points ($n = 100$).

b, Centrosomes treated previously with CSF-released extract, thus containing disengaged centrioles, were incubated with cycling extract for the times indicated. Centrosomes at each time point ($n = 50$) were visualized by anti-centrin (left of each panel, green in merged image) and anti-human pericentrin (middle of each panel, red in merged image) antibodies. The percentage of doublets is shown in the upper left of the first panel of each time point; percentage of singlets in lower right. Scale bars: $1 \mu\text{m}$.

duplication in the same cycle (Supplementary Fig. 2). This strategy for controlling centrosome duplication closely resembles that for DNA replication²¹; for both processes it is the absence of licensing activity until the end of mitosis that limits replication/duplication to once per cell cycle.

Our results suggest that centriole disengagement requires the activity of separase, a protease previously shown to release sister-chromatid cohesion in anaphase². This remarkably parsimonious design temporally links these two essential events in the cell cycle. The timing of these events is critical: premature sister chromatid separation results in mis-segregation of chromosomes², and premature centriole disengagement potentially leads to multipolar spindles, also a source of abnormal divisions^{3,4}. Our experiments made use of the simple embryonic cell cycle; however, recent results also suggest a link between premature activation of separase and centriole disengagement in somatic cells. For example, the Tax viral oncoprotein prematurely activates APC and causes multipolar spindles^{22,23}; we suggest that this might occur by premature APC-mediated activation of separase. Similarly, the premature centriole disengagement that occurs in some cells entering mitosis with DNA damage³ might be explained by separase activation that accompanies DNA repair²⁴. We note that we have not yet directly tested the role of separase by depletion from extract or cells, and it is formally possible that the two mechanistically distinct treatments used here block an activity other than separase required for disengagement. Direct tests are complicated by the pleiotropic phenotype associated with separase

depletion²⁵. Recent development of conditional null mutations in mouse^{26,27} might allow a direct test, but will require more detailed experiments than those presented.

Many human cancers exhibit centrosome amplification, which is strongly correlated with genomic instability²⁸. The simple model for centrosome duplication described here serves as a framework upon which other regulatory modules can be added in somatic cells, and it will be of interest to determine how the mechanism is subverted in cancer cells.

METHODS

Details of cell culture, antibodies, centrosome isolation and *Xenopus* extract preparation are described in Supplementary Information.

In vitro centriole disengagement assay. Forty microlitres of the CSF extract was incubated with $1 \mu\text{l}$ purified centrosomes (approximately 2.5×10^4) at 23°C for 5–10 min, and calcium was added to trigger centriole disengagement. Where indicated, extract was incubated with added components for 30 min on ice or at 23°C before the addition of centrosomes. Reactions were stopped at the indicated time after calcium addition by the addition of $1 \mu\text{l}$ of 0.5 M EDTA, and frozen in liquid N_2 . To assay DNA morphology, sperm nuclei were added, fixed at time points, and labelled with Hoechst 33258. The presence or absence of sperm chromatin had no effect on the centriole disengagement activity. To immunostain centrosomes, $1\text{--}2 \mu\text{l}$ of extract was squashed between slide and coverslip (12 mm round) and frozen by immersion in liquid N_2 for 30 s. The slide was removed from liquid N_2 and the coverslip removed by prying off with a single-edge razor blade, leaving a thin layer of frozen extract. These samples were fixed in 100% methanol for 5 min at -20°C , followed by re-hydration in PBS.

After incubation with primary and secondary antibodies, samples were dehydrated in 100% methanol, and cleared and mounted in benzyl alcohol/benzyl benzoate clearing solution as described previously¹¹. Note that anti-human C-Nap1 and anti-human pericentrin antibodies react specifically with the human proteins, and not *Xenopus* (not shown), thus only the input human centrosomes are labelled. In experiments in which centrin–GFP-labelled centrosomes were used, centrioles were visualized directly using fluorescence microscopy.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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**THE CAREERS
MAGAZINE FOR
SCIENTISTS**

With insufficient homegrown talent entering key disciplines such as engineering and the physical sciences, the United States has long relied on foreign graduates to make up its PhD shortfall. But since the terrorist attacks of 11 September 2001, the number of international students enrolling in US graduate programmes has dropped dramatically. As a result, numerous initiatives have been launched in a bid to attract students back to the country. And, according to a report issued by the US Council of Graduate Schools, these efforts are at last beginning to bear fruit.

The report shows that, compared with last year, 2006 has seen an 11% increase in applications from foreign graduates and a 12% increase in the number of positions being offered (see www.cgsnet.com). But the key figure — how many students actually enrol — will not be available until October.

The year-on-year decline in enrolment was the result of several factors, many of which are linked to US policy. The tightening of security in the wake of the terrorist attacks caused significant problems for foreign students trying to get visas. Even those who already had visas faced considerable difficulties re-entering the United States from their home countries. The fresh focus on defence-related research has also meant that the US budget for basic research and development has remained relatively flat — or has even declined for some disciplines — making the country less attractive. At the same time, other nations including Britain and Canada have actively wooed foreign graduate students.

But, as the new report explains, these issues have spurred US universities into action: 79% of admissions offices surveyed said that they had implemented at least one new practice to entice foreign graduate students to their institution. These initiatives include dedicated staff for recruiting and assisting the students, special funding schemes and international collaborations. If the US labour market is to avoid losing its competitive edge in science, then the decline in enrolments must be reversed. The latest statistics may provide a glimmer of hope, but there's still a lot of work to be done.

Paul Smaglik, *Naturejobs* editor

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Speak, geek

Every dog will have its day.

Eileen Gunn

People call me a nerd, but I say I'm a geek. In my youth, I ran wild on a farm and bit the heads off chickens. This was before the Big Tweak, back when a chicken was dinner, and a dog was man's best friend.

They call me a mutt, too. Sure, I'm a mutt. Mutt is good. Mutt is recombinant DOG. And I'm a smart mutt. I was smart before they tweaked me, and I'm a hell of a lot smarter now.

I've watched untweaked bitches (pardon the expression) trot by on leashes. I don't envy them. I don't even want to breed with them. (And, yes, I am quite intact, not that you were asking.) Their days are filled with grooming and fetching and the mutual adoration that comes with being someone's trophy pet. I have a second life, a life of the mind, beside which theirs pales.

Not that I take credit for my enhancements. Didn't get a choice. But gene engineering is inherently fascinating. Massively multiplayer, fraught with end-of-life-as-we-know-it threats. It made me who I am. I've chosen it for my career.

Working at the Lazy M is the job of a lifetime. Loyalty is a big thing here, and you'd better believe I deliver. I love this place so much that I don't want to go home at night. There's free kibble and a never-empty water dish right outside my kennel. (Did I tell you we each get our own private kennel? Except for the contractors, of course.)

I understand my place in the corporate structure, and my importance to the Man update.

There's always more code in the genome — always something to snip or interpolate. That's why I was there in the middle of the night: a last round of corrections before the code freeze on Man 2.1.

I was taking a good long slurp of water when I noticed the cats. They weren't making a big deal of it — just quietly going about their business — but there were cats in all the cubicles, in the exec offices, in the conference rooms. It looked like they were running a whole separate company in the middle of the night.

Who hired them? HR doesn't hire cats for R&D. They're not task oriented, or good at working within a hierarchy. They sleep all day. Better suited to industrial espionage.

Back on the farm, I was a watchdog, and I've still got a bit of that energy. Better keep an eye out, I think. So I'm lying there in the doorway to my office, nose on my paws, like I'm taking a break, when the alpha cat comes by. Big muscular Siamese mix. His flea collar says 'Dominic' in red letters.

"Hey, Dominic," I call. I feel like a character in *The Sopranos*. You ever see that show? No dogs to speak of, but lots of food. Great food show.

The cat stops. Stares. "You talking to me?"

"What's the story here, Dominic?"



"No business of yours." He narrows his weird cat eyes, then yawns ostentatiously. He turns away, shows me his butt, and walks slowly off, his loose belly-fur swaying. I notice that his ears are facing backwards, in case I rush him: he's not as nonchalant as he appears.

Detective work is needed. I go down to the cafeteria, keeping my eyes open en route. Funny thing: I notice there are cats in and out of Susan Gossman's office like she had a catnip rug. Gossman? Seen her in the hallways. We'd never spoken. More of a cat person.

I slip a few bucks in a vending machine for one of those big leather bones. I chew.

When I get back to my office a savvy-

looking brunette in a well-cut suit is sitting on a corner of the desk. Gossman. "You're wondering about the cats," she says.

I wave my tail a bit. Not a wag, but it says I'm paying attention. Her hair has copper highlights. Or maybe she put drugs in my water dish.

"Project Felix," she says, "is an undocumented feature of the new Man release."

"Undocumented is right," I say. "You're doing some kind of super-tweaking with the human-cat chimaeras, and I don't think it's for Man 2.1. Chimaeric DNA ripping through the wild? Influenza vector?"

"You're a smart pup," she says.

My hackles raise. "Do Bill and Steve know what you're doing?"

"Down, boy," says Gossman. Instinctively, I sit back on my haunches. "Bill and Steve will find out soon enough. This is all for the better. Infected humans — and dogs too — will be smart and independent. The rest will just keep right on dipping seafood feast into plastic bowls."

Woof. That's straightforward.

She looks at me speculatively. "Right now, we need a top-flight coder."

I'm alert: my nose is quivering.

But Gossman is relaxed. "Everybody knows dogs are the best. But, as a dog," she says, "you have some loyalty issues. Am I right?"

I just stare at her.

"Loyalty is a gift, freely given," says Gossman.

I give a half-hearted wag of my tail. Not for dogs, I think.

"But not for dogs," says Gossman. "Wouldn't you

like the freedom to make your own decisions? A whiff of feline flu could make all the difference." She pulls a tiny aerosol can out of her purse.

I've got reflexes humans can't compete with. I could have it out of her hand in a split second. But do I owe my loyalty to the company, or to the great web of which all dogs, cats and humans are part?

She sprays. I breathe deep. She's right: dogs are the best coders. ■

Eileen Gunn lives in Seattle. Her short-story collection *Stable Strategies and Others* (2004) was shortlisted for the Philip K. Dick, James Tiptree Jr and World Fantasy awards. She is editor/publisher of the *Infinite Matrix* (www.infinitematrix.net) and is on the board of directors of the Clarion West Writers Workshop.

JACEY